



Porcine Intestinal Disease Complex in nursery pigs, with special focus on the prudent use of antimicrobials

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Porcine Intestinal Disease Complex in nursery pigs, with special focus on the prudent use of antimicrobials

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Table of contents

PREFACE.....	VI
ACKNOWLEDGEMENTS.....	VII
LIST OF ABBREVIATIONS.....	IX
SUMMARY	X
SAMMENDRAG	XII
LIST OF FIGURES.....	XIV
LIST OF TABLES.....	XV
1 INTRODUCTION	1
1.1 Motivation	1
1.2 Project purpose and aim	2
1.3 Outline of the thesis	2
2 BACKGROUND.....	3
2.1 Prudent use of antimicrobials in veterinary medicine	3
2.1.1 Antimicrobial resistance	3
2.1.2 Implementation of prudent use.....	4
2.1.3 Prudent use in Danish pig production	5
2.1.4 Project definition of prudent use	7
2.2 Intestinal diseases in nursery pigs	7
2.2.1 Description of diarrhoea outbreaks in nursery pigs.....	7
2.2.2 Single-pathogen-related intestinal diseases in nursery pigs	9
2.2.3 Causative agents in diarrhoea outbreaks in Danish nursery pigs	12
2.2.4 Risk factors for intestinal disease.....	13
2.2.5 Economic impact	14
2.2.6 Causative relationships between pathogens and intestinal diseases.....	15
2.3 Porcine intestinal disease complex	15

2.4 Diagnosis and treatment of intestinal diseases	17
2.4.1 Diagnosis of intestinal diseases.....	17
2.4.2 Diagnosing PIDC by testing pen floor samples	21
2.4.3 Antimicrobial treatments in pigs	23
2.4.4 Antimicrobial treatment of intestinal diseases	25
 3 STUDY OBJECTIVES AND HYPOTHESIS.....	 29
 4 MATERIALS & METHODS	 31
4.1 Study designs.....	32
4.2 Description of datasets.....	32
4.3 Sample size considerations	35
4.4 Randomisation	36
4.5 Data collection.....	38
4.6 Antimicrobial treatments	41
4.7 Laboratory analysis	41
4.8 Data management and statistical analysis	43
 5 RESULTS.....	 46
5.1 Results of Study 1.....	46
5.1.1 Diarrhoea prevalence in nursery pigs.....	46
5.1.2 Prevalence of intestinal pathogens	47
5.2 Results of Study 2.....	48
5.2.1 Descriptive results Study 2	49
5.2.2 Effect of treatment strategy and type of antimicrobial on average daily weight gain	52
5.2.3 Effect of antimicrobial agent on diarrhoea prevalence and excretion of pathogens.....	52
5.2.4 Effect of demonstrating faecal bacterial intestinal pathogens at antimicrobial treatment initiation on the average daily weight gain	53
5.3 Results of Study 3.....	53
5.3.1 Agreement among three different diagnostic approaches for detecting ETEC	54
5.3.2 Antimicrobial susceptibility testing	55

6 PAPERS	56
6.1 Paper I	57
6.2 Paper II.....	64
6.3 Paper III	73
6.4 Paper IV.....	81
7 DISCUSSION	102
7.1 Discussion of materials & methods	102
7.2 Discussion of hypothesis 1: New objective methods to identify intestinal infections in nursery pigs are necessary to ensure the prudent use of antimicrobials	105
7.3 Discussion of hypothesis 2: Efficacy of batch treatment depends on the correct timing and choice of antimicrobial	107
7.4 Discussion of hypothesis 3: Testing of pen floor samples may enhance the prudent use of antimicrobials for batch treatment of intestinal infections in nursery pigs	108
7.5 Discussion of hypothesis 4: Intestinal infections in batches of nursery pigs are often of mixed aetiology	110
8 CONCLUSION.....	112
9 IMPLICATIONS AND PERSPECTIVES	114
9.1 Future perspectives.....	114
9.2 Practical importance of the results.....	114
10 REFERENCES	115

Preface

This PhD thesis is intended to fulfil the requirement for the PhD degree at the University of Copenhagen, Faculty of Health and Medical Sciences, Department of Animal and Veterinary Sciences, Denmark. The scholarship was awarded by the Faculty of Medical Sciences at the University of Copenhagen. The studies were conducted in collaboration with SEGES Danish Pig Research Centre.

Nicolai Rosager Weber, June 2017

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List of abbreviations

ADD	Average daily doses
ADG	Average daily weight gain
AMR	Antimicrobial resistance
ETEC	Enterotoxigenic <i>Escherichia coli</i>
IHC	Immunohistochemistry
MIC	Minimum inhibitory concentrations
PCV2	Porcine circovirus type 2
PE	Proliferative enteropathy
PED	Porcine epidemic diarrhoea
PIDC	Porcine Intestinal Disease Complex
PWD	Post-weaning diarrhoea
qPCR	Quantitative Polymerase Chain Reaction
SPF	Specific pathogen free
TGE	Transmissible gastroenteritis

Summary

Antimicrobial use in food animals attracts major scientific and public attention due to the risk of transmitting antimicrobial resistance (AMR) to the human population. The prudent use of antimicrobials (including the initiation of treatment based on precise diagnosis) can reduce the risk of AMR development. The majority of antimicrobials prescribed in Danish pig production are for the treatment of intestinal diseases in nursery pigs, for which infection by enterotoxigenic *E. coli* (ETEC), *L. intracellularis*, and *B. pilosicoli* are considered to be the major causes, and mixed infections are common. The term Porcine Intestinal Disease Complex (PIDC) is proposed in this thesis to describe diseases of multiple aetiology affecting the small and large intestines, causing clinical disease and failure to gain weight.

The aim of this thesis was to develop new objective methods for assessing the intestinal health of nursery pigs at batch level, and to improve treatment strategies in order to achieve prudent antimicrobial use through more precise and potentially reduced antimicrobial treatment.

This PhD project consists of three studies conducted in Danish commercial pig herds. Study 1 is a prevalence study in 16 pig herds, evaluating whether the clinical assessment of groups of nursery pigs and visual assessment of diarrhoeic faecal droppings on the pen floor can be used as a method to identify diarrhoeic nursery pigs with intestinal infections. Study 2 is a field trial assessing the efficacy of antimicrobial treatment regimens of PIDC in nursery pigs. Four treatment strategies and two types of antimicrobials were tested in a 2x4 factorial design. Study 3 is a study of ETEC isolated from both diarrhoeic nursery pigs and from pen floor samples. It assesses the diagnostic value of analysing pen floor samples for the detection of ETEC-positive diarrhoeic pigs and for selecting antimicrobial agents.

Study 1 showed that the diarrhoeic status of the individual pig and diarrhoeic faecal droppings in pens was a poor indicator of intestinal infections with ETEC, *L. intracellularis* and *B. pilosicoli*, and that subclinical infections were common. The results of Study 2 showed that the highest average daily weight gain (ADG) was achieved when treatment was initiated 14 days after weaning in pens where intestinal pathogens were detected on the pen floor. Doxycycline was more effective in reducing diarrhoea and *L. intracellularis* excretion levels than treatment with tylosin. The results of Study 3 showed that both bacterial culture and quantitative Polymerase Chain Reaction (qPCR) testing of pen floor samples can be used as a diagnostic approach for detecting groups of ETEC-positive diarrhoeic nursery pigs. Furthermore, when the resistance

profiles of isolates from the same pen (pen floor and pig samples) were compared, an excellent agreement was observed for all clinically relevant antimicrobial agents used for the treatment of ETEC-infected pigs. Therefore, susceptibility testing of ETEC isolates from the pen floor could represent a convenient method for testing resistance and selecting antimicrobial agents.

In conclusion, the results of these studies have contributed new knowledge about the diagnosis and treatment of PIDC in nursery pigs. qPCR testing of pen floor samples can be used to support the decision to initiate antimicrobial treatment of PIDC in groups of nursery pigs before clinical signs are evident. Furthermore, susceptibility testing of isolates from pen floor samples is suggested as a new approach for selecting antimicrobial agents.

Sammendrag

Brugen af antibiotika til produktionsdyr tiltrækker stor offentlig og videnskabelig opmærksomhed på grund af risikoen for overførsel af antibiotika resistens til den humane population. For at reducere risikoen for udvikling af antibiotika resistens, skal anvendelsen af antibiotika i produktionsdyr være ansvarlig. Dette indebærer at enhver indledning af behandling skal være baseret på en præcis diagnose. Størstedelen af antibiotika anvendt i dansk svineproduktion bliver udskrevet til behandling af tarmsygdomme hos smågrise, hvor infektioner med enterotoxiske *E. coli* (ETEC), *L. intracellularis* og *B. pilosicoli* anses for at være hovedårsagerne og blandede infektioner er almindelige. I denne afhandling foreslås udtrykket Porcine Intestinal Disease Complex (PIDC) til at beskrive sygdomme i tynd- og tyktarmen af multipel infektiøs ætiologi, der forårsager klinisk sygdom og reduceret tilvækst.

Formålet med denne afhandling var at udvikle nye objektive metoder til vurdering af tarmsundhed hos smågrise på sektionsniveau og forbedre behandlingsstrategier for at opnå et mere præcist, rationelt og mulig reduceret antibiotikaforbrug.

Dette PhD projekt består af tre studier udført i danske kommercielle svinebesætninger. Studie 1 er et prævalensstudie i 16 svinebesætninger der evaluerer om klinisk vurdering af grupper af smågrise og visuel vurdering af diarreklatter i stibunden kan bruges som en metode til at identificere diarré-grise med intestinale infektioner. Studie 2 er et felt forsøg, der vurderer effekten af antibiotikabehandlingsregimer af PIDC hos smågrise. Fire behandlingsstrategier og to typer antibiotika blev testet i et 2x4 faktor design. Studie 3 er en undersøgelse af ETEC isoleret fra diarrégrise og fra stibundsprøver, hvor den diagnostiske værdi af at analysere stibundsprøver til påvisning af ETEC-positive diarrégrise og valg af antibiotika blev vurderet.

Studie 1 viste, at diarrestatus hos den enkelte gris og diarreklatter i stibunden var en dårlig indikator for intestinale infektioner med ETEC, *L. intracellularis* og *B. pilosicoli* og at subkliniske infektioner var almindelige. Resultatet af Studie 2 viste, at den højeste gennemsnitlige daglige tilvækst blev opnået, når behandling blev påbegyndt 14 dage efter fravænning i stier, hvor intestinale patogener blev påvist i stibunden. Doxycyklin var mere effektivt til at reducere diarre og udskillelsesniveauet af *L. intracellularis* end behandling med tylosin. Resultatet af Studie 3 viste, at både dyrkning og qPCR analyse af stibundsprøver kan bruges som en diagnostisk fremgangsmåde til påvisning af grupper af ETEC-positive diarrégrise. Endvidere blev der observeret en god overensstemmelse for alle kliniske relevante antibiotika til behandling af ETEC-smittede grise, når

resistensprofilerne fra isolater fra den samme sti (stibund og griseprøver) blev sammenlignet. Derfor kan følsomhedstest af ETEC-isolater fra stibunden være en brugbar metode til resistensbestemmelse og derved valg af antibiotika.

Resultaterne af disse undersøgelser har bidraget med ny viden om diagnostik og behandling af PIDC hos smågrise. qPCR analyser af stibundsprøver kan anvendes til at understøtte beslutningen om indledning af antibiotika behandling af PIDC i grupper af smågrise, inden kliniske tegn er tydelige. Endvidere foreslås resistensbestemmelse af ETEC isolater fra stibundsprøver som en ny metode til valg af antibiotika.

List of figures

Figure 1	Interaction of infectious, dietary and management factors that result in Porcine Intestinal Disease Complex (PIDC)	16
Figure 2	Illustration of sampling and treatment procedure in one batch in Study 2	39
Figure 3	Overview of sample procedure at pen level in Study 3	40
Figure 4	Plot of diarrhoea prevalence by age of pig	47
 <i>Paper III</i>		
Figure 1	Overview of sample procedure at pen level	76

List of Tables

Table 1	Distribution of pens by antimicrobial and strategy group after randomisation in Study 2	34
Table 2	List of clinical registrations used in Study 1 for the examination of selected pigs	38
Table 3	Antimicrobial concentration ranges and resistance breakpoints used in Study 3 for susceptibility testing of <i>E. coli</i> isolated from faecal samples from weaned pigs and pen floors	42
Table 4	Overview of dataset included in the thesis	44
Table 5	Overview of statistical analyses performed for each dataset and specific study objectives	45
Table 6	Results of the clinical examination of 2,866 pigs	46
Table 7	Distribution of pathogen detection in random sampled non-medicated pigs, stratified by herd, batch, pen, and pig level	48
Table 8	Description of Herd A, B & C	49
Table 9	Results of univariable analyses of daily weight gain and explanatory variables	49
Table 10	Mean pen-level diarrhoea prevalence in pens not subjected to antimicrobial treatment	50
Table 11	Prevalence of pathogen-positive samples detected in pen floor samples by qPCR in pens not subjected to antimicrobial treatment	51
Table 12	Distribution of sampled <i>E. coli</i> isolates by batch	53
Table 13	Comparison of diagnostic approaches for detecting ETEC in pen floor and rectal faecal samples by bacterial culture and PCR or quantitative PCR	54
Table 14	Overview of resistance in ETEC isolates from pig and pen floor samples	55
Paper I		
Table 1	Clinical findings other than diarrhoea in 3,060 nursery pigs during clinical examination by visual inspection	60
Table 2	Results of qPCR analysis for <i>Escherichia coli</i> F4 and F18, <i>Brachyspira pilosicoli</i> and <i>Lawsonia intracellularis</i> in 256 faecal samples from nursery pigs	61
Table 3	Simultaneous presence of pathogens <i>Escherichia coli</i> F14 and F18, <i>Lawsonia intracellularis</i> , <i>Brachyspira pilosicoli</i> in 256 faecal samples from nursery pigs	61
Table 4	Detection of one or more pathogens by level of diarrhoeic pools from where the pigs were housed	61
Table 5	Excretion levels of intestinal pathogens in positive qPCR samples from 256 nursery pigs	62
Paper II		
Table 1	Distribution of pens per herd and per treatment strategy	66
Table 2	Detection and excretion levels of pathogens in faecal pen floor samples by qPCR, and diarrhoea prevalence in non-treated pens	68
Table 3	Total bacterial load of <i>L. intracellularis</i> and <i>E. coli</i> F18	69
Table 4	Estimates for fixed effects, intraclass correlation coefficients (ICC) for random effects and estimated means from a linear mixed model for average daily weight gain from 14 to 35 days after weaning (kg)	69
Table 5	Faecal excretion of <i>Lawsonia intracellularis</i> analysed by qPCR from pooled pen floor samples	70
Table 6	Estimates for fixed effects, intraclass correlation coefficients (ICC) for random effects and estimated means from a linear mixed model for average daily weight gain from 14 to 35 days after weaning (kg) in pigs selected for Treatment Strategy 1 (S1)	70

Paper III

Table 1	Adhesin and toxin genes detected in 380 <i>E. coli</i> isolates from rectal samples of diarrhoeic pigs and pen floor samples 14 - 28 days post-weaning	77
Table 2	Quantitative PCR results for <i>E. coli</i> fimbrial genes F4 and F18, <i>Lawsonia Intracellularis</i> and <i>Brachyspira Pilosicoli</i> from 31 pen floor samples	78
Table 3	Relationship between the presence of virulence factor genes and haemolysis in <i>E. coli</i> isolates	78
Table 4	Comparison of diagnostic approaches for detecting ETEC in pen floor and rectal faecal samples by bacterial culture and PCR or quantitative PCR	79

Paper IV

Table 1	Antibiotic concentration ranges and resistance breakpoints used for susceptibility testing of <i>E. coli</i> (n = 380) isolated from faecal samples from weaned pigs and pen floors	96
Table 2	Distribution of MICs and occurrence of resistance in 89 ETEC and 291 Non-ETEC isolates	97
Table 3	Distribution of resistant isolates at herd level	98
Table 4	Profile of antimicrobial resistance in <i>E. coli</i> isolates in faecal samples from weaned pigs and pen floors	99
Table 5	Resistance patterns in ETEC isolates detected from pig and pen floor samples in the same pen	100
Table 6	Agreement in the resistance classifications of ETEC isolates for selected antimicrobial agents between pig and pen isolates	101

1 Introduction

1.1 Motivation

Antimicrobial use in food animals has attracted major public and political attention in recent years. The risk of spreading antimicrobial resistance (AMR) from food animals to the human population has been the main driver for promoting a more prudent use of antimicrobial in food animals.

Antimicrobials prescribed for pigs account for 75% of the total usage (81.5 tonnes of active compound in 2015) for animals in Denmark. Approximately one-third of the antimicrobials prescribed for pigs are used for oral batch treatment of intestinal diseases in nursery pigs. Antimicrobials prescribed for gastrointestinal diseases in nursery pigs accounted for 62.2% of the total Average Daily Doses (ADD) of antimicrobials prescribed for pigs in 2015. Since 2010, Danish authorities have issued government-set thresholds for antimicrobial usage at farm-level – known as the Yellow Card initiative. The threshold has been lowered four times since the scheme was implemented. Furthermore, there is a national target of a 15% reduction in the use of antimicrobials in pigs between 2015 and 2018, which would amount to 11.9 tonnes of active compound (DANMAP, 2016). Preventive methods such as health-promoting diets and vaccinations to reduce the incidence of intestinal disease are important aspects in achieving the targeted reduction in antimicrobial usage. Another key element is to develop and test new strategies for the most effective antimicrobial treatments in nursery pigs suffering from intestinal diseases. It is important to establish diagnostic decision-support tools for farmers and pig practitioners to aid in the identification of groups of infected pigs requiring antimicrobial treatment among groups of healthy pigs for which treatment can be avoided.

My enrolment as a PhD student began on 1st April 2013 at the University of Copenhagen, within the Faculty of Health and Medical Sciences, Department of Large Animal Sciences. Funding for the present PhD project was provided by the Danish Pig Levy Fund (Svineafgiftsfonden). During my PhD, I have worked part-time as a pig practitioner at Landbrugets Veterinære Konsulentjeneste (LVK).

This experience as a pig practitioner contributed to my own motivation for this project, as I find the subject of antimicrobial treatment of intestinal diseases highly relevant for farmers and

veterinarians. Current diagnostic tools and the prevalence of intestinal infections challenge pig veterinarians when a decision about the treatment of groups of pigs must be made. In addition, current methods make it difficult to identify healthy groups of pigs with no need of antimicrobial treatment. I was keen to find new ways to help farmers and veterinarians with this challenge and achieve a more prudent use of antimicrobials in nursery pigs.

1.2 Project purpose and aim

The purpose of the PhD project was to improve intestinal health and thereby reduce feed costs in nursery pigs by refining the diagnosis and treatment of intestinal diseases.

The aim was to develop new objective methods for assessing porcine intestinal health at batch level and to improve treatment strategies to achieve a prudent use of antimicrobials through a more precise and potentially reduced antimicrobial use.

1.3 Outline of the thesis

Chapter 2 contains an introduction to the prudent use of antimicrobials in veterinary medicine. Furthermore, intestinal diseases in nursery pigs are described, with a focus on causative agents, diagnostic methods (including susceptibility testing), and antimicrobial treatment regimens.

Chapter 3 includes the hypothesis and study objectives of the project.

Chapter 4 describes the materials and methods of the project in order to provide an overview of the studies.

Chapter 5 presents the main results obtained in the studies.

Chapter 6 contains the four papers of the project:

Paper I: Occurrence of diarrhoea and intestinal pathogens in non-medicated nursery pigs.

Paper II: Batch medication of intestinal infections in nursery pigs - A randomised clinical trial on the efficacy of treatment strategy, type of antibiotic and bacterial load on average daily weight gain.

Paper III: Comparison of bacterial culture and qPCR testing of rectal and pen floor samples as diagnostic approaches to detect enterotoxigenic *Escherichia coli* in nursery pigs.

Paper IV: Comparison of antimicrobial resistance in *E. coli* isolated from rectal and floor samples in pens with diarrhoeic nursery pigs.

Chapters 7, 8 and 9 contain a general discussion, conclusion and perspectives of the project.

2 Background

2.1 Prudent use of antimicrobials in veterinary medicine

In this thesis, the prudent use of antimicrobials is considered to be synonymous with antimicrobial stewardship or responsible use. Several definitions of prudent use have been suggested, but in general, it describes the practice of reducing the development, maintenance and spread of antimicrobial resistance (AMR) through the optimal selection of antimicrobials, dosage and duration of treatment, as well as minimising misuse and overuse (Weese, 2006).

The World Health Organization (WHO) defines the prudent use of antimicrobials as usage that maximises therapeutic effect to protect both animal and human health, while minimising the development of AMR (WHO, 2012).

The World Organisation for Animal Health (OIE) has defined the prudent use of antimicrobials in veterinary medicine as practical measures and recommendations to preserve and maintain the therapeutic efficacy of antimicrobials for the benefit of animal and public health (OIE, 2016). Prudent use of antimicrobials is defined by the European Commission as a rational and targeted use, maximising the therapeutic effect and minimising the development of AMR. Due to the risk of co-resistance and spread of AMR to the human population, prudent use should result in antimicrobials only being used when necessary, thereby reducing the overall use (European-Commission, 2015). However, the European Platform for the Responsible Use of Medicines in Animals (EPRUMA) defines prudent use as maintaining efficacy and preventing and minimising adverse reactions in animals (EPRUMA, 2008).

A common feature of the definitions from WHO, OIE and the European Commission is the aspect of “one health”, where prudent use has an impact on both animal and human health, since the development of AMR in animals can also have an impact on the human population. This should be considered when the prudent use of antimicrobials is implemented.

2.1.1 Antimicrobial resistance

As mentioned, two aspects of AMR development must be considered: the development of AMR in animal-specific pathogenic strains, and the transmission of resistance from animal to human populations.

Any antimicrobial treatment of animals can lead to the development of AMR (Burch et al., 2008; Marshall and Levy, 2011). This will result in a reduced treatment efficacy that can make it more

difficult to control infectious diseases in animals. The consequences include decreased animal health and welfare with high economic losses (Bengtsson and Greko, 2014). Reduced efficacy of an antimicrobial agent can lead to higher consumption or a shift to a more potent broad-spectrum agent. Most of the antimicrobial agents used in veterinary medicine were discovered decades ago, and the majority of newly discovered molecules with antimicrobial effects are reserved for human medicine (Shryock, 2004). As a consequence, future control of contagious diseases in food animals must be based on prevention and prudent antimicrobial use in order to ensure animal health.

Much attention has been paid to the transmission of resistance from food animals to the human population (van den Bogaard and Stobberingh, 2000; Marshall and Levy, 2011), and several routes of transmission have been described. One example is AMR in zoonotic bacteria such as *Salmonella spp.* and *Campylobacter spp.*, which can be directly transmitted via the food chain and cause infections in humans (Bengtsson and Greko, 2014). Resistance in zoonotic bacteria is lower in countries with restrictive policies on antimicrobial use in food animals (Garcia-Migura et al., 2014). Another example is the transmission of AMR bacteria via direct contact with live animals. Methicillin resistant *Staphylococcus aureus* (MRSA) has attracted a lot of attention due to the discovery of the transmission route between livestock-associated MRSA (cc398)-positive pig herds and farm personnel (Grontvedt et al., 2016). A third AMR transmission route is through commensal bacteria in animals, for example, *E. coli* and enterococci, which may be pathogenic in humans. AMR in reservoirs of commensal enteric bacteria with animal origins can be spread via plasmid-based gene transmission to the human population (Laxminarayan et al., 2013). Direct evidence of this transmission pathway has been difficult to obtain, but indirect evidence has been exemplified by the discovery of genes resistant to the antimicrobial streptothricin in *E. coli* isolated from farmers. Streptothricin was only used as a growth promoter in food animals, suggesting the possibility of such a transmission pathway (Witte, 2000).

2.1.2 Implementation of prudent use

In order to implement the prudent use of antimicrobials in humans and food animals, guidelines have been developed by national, European and international bodies (Teale and Moulin, 2012; European-Commission, 2015). These guidelines describe practical measures and recommendations that will benefit animal and public health, and will preserve the therapeutic efficacy of antimicrobials. Furthermore, the responsibilities of all stakeholders involved in the process of antimicrobial use and regulation are described. In the OIE Terrestrial Code, the

veterinarian's responsibilities include the prevention, identification and treatment of animal diseases, as well as the promotion of both public and animal health and animal welfare (OIE, 2016). In order to safeguard animal and human health by reducing AMR, the European Food Safety Authority has recently pinpointed three key elements (EFSA, 2017):

1. REDUCE: Antimicrobials have to be reduced by setting national targets for usage, increasing the responsibility of veterinarians, and using antimicrobials only when needed. To ensure their rational and targeted use, antimicrobials should only be administered or prescribed when necessary, and the choice of antimicrobial agents should be based on clinical experience and diagnostic laboratory information, with pathogen isolation, detection and susceptibility testing where possible, leading to the selection of antimicrobial agents with as narrow a spectrum as possible (OIE, EU). Furthermore, the use of critically important antimicrobials for preventing or treating life-threatening infections in humans should be minimised in veterinary medicine.

2. REPLACE: Alternatives to antimicrobials (prebiotics, probiotics, organic acids etc.) must be considered whenever possible. Furthermore, in order to replace antimicrobials, research studies on new alternatives or methods to reduce antimicrobial treatment are necessary. It is also necessary to improve the legal framework of the EU to facilitate the development and authorisation of new alternatives to antimicrobial treatments.

3. RETHINK: The need for antimicrobial treatments can be reduced by better farming practices, improved external biosecurity (to prevent the introduction of diseases to herds), improved health and welfare of animals, protection from diseases through vaccines, or by breeding disease-resistant animals. Farm systems with high antimicrobial usage should be evaluated, and alternative farming systems with lower antimicrobial usage should be investigated. Finally, awareness of AMR should be addressed through educating veterinarians and farmers.

2.1.3 Prudent use in Danish pig production

When discussing the prudent use of antimicrobials in food animals in different countries, the considerable differences between national regulations must be taken into account. In Denmark, restrictions on antimicrobial use in pig production have applied for over 25 years and are regulated by government ruling and by voluntary initiatives by the pig industry. Any antimicrobials used for the treatment of pigs can only be prescribed by authorised veterinarians after clinical inspection of the herd, batch or animal, and veterinarians are not allowed to benefit economically from antimicrobial prescriptions (1996). Other restrictions include the development of guidelines for antimicrobial use (1996), a voluntary end to the use of growth

promoters (1999), national monitoring of antimicrobial use and resistance in food producing animals (Danmap, 2000), restrictions on the use of critically important antimicrobials (Flourquinolons, 2002, 3rd/4th gen. Cephalosporin, 2010, Tetracyclines 2016, Colistin 2017), the Yellow Card initiative, defining the maximum herd-level use of antimicrobials (2010), national targets for the reduction of antimicrobial use (2010, 2015) and legislation requiring laboratory diagnostic documentation in relation to group medication for enteric and respiratory diseases (2014). The result of these restrictions, regulated by government ruling and voluntary initiatives by the Danish pig industry, has been a very low usage of critically important antimicrobials and an overall reduction in annual consumption from approximately 160 tonnes in 1997 (therapeutic + growth promoters) to 81.5 tonnes in 2015, while pig production has increased considerably in the same period. After the introduction of the Yellow Card initiative, the annual consumption in pig production was reduced by 22% between 2009 and 2015 (DANMAP, 2016).

The focus for achieving prudent use in Danish pig production has thereby mainly been on restricting use to veterinary prescription only, therefore reducing the overall antimicrobial usage and restricting the use of critically important antimicrobials.

As a result of these strict regulations, antimicrobials have become a limited resource, increasing the need for accurate and precise diagnostic work by pig veterinarians. A key element in achieving prudent use is the development of new and precise diagnostic tools in veterinary pig practice, in order to decide whether antimicrobial treatment is necessary or can be avoided. Furthermore, if antimicrobial treatment is deemed necessary, the next step is to select an appropriate antimicrobial (EPRUMA, 2008). Antimicrobial selection should be based on on-farm sampling and testing of isolates, as well as the results of national resistance surveillance and monitoring. Furthermore, development of preventive methods – such as effective vaccines against the bacterial pathogens responsible for the majority of antimicrobial treatments, or health promoting diets – are essential to achieve an overall reduction in antimicrobial use (Teale and Moulin, 2012). An investigation of Danish pig herds reporting a decrease of $\geq 10\%$ in antimicrobial use after the introduction of the Yellow Card initiative showed the decrease was achieved through a combination of increased use of vaccines and reduced group medication (Dupont et al., 2017).

2.1.4 Project definition of prudent use

The aim of this project was to develop new objective methods for assessing porcine intestinal health at batch level and to improve treatment strategies to achieve a prudent use of antimicrobials.

As described by EFSA, prudent use can be achieved by *reducing* the overall use, *replacing* antimicrobials with other treatment options, and *rethinking* production in order to reduce the need for antimicrobial treatments. The main elements in this project focussed on improving the diagnosis and treatment of intestinal diseases. Although replacing the need for antimicrobials by preventive measures and rethinking pig production are also essential to achieving prudent use, they were not included in this project and are not discussed further. Therefore, the term ‘prudent use’ is used in this thesis to describe precise and accurate antimicrobial treatments that result in efficient treatment with a potentially lower usage.

2.2 Intestinal diseases in nursery pigs

Along with respiratory diseases, those affecting the intestines of pigs are the most common diseases in modern pig production worldwide (Thomson and Friendship, 2012). In the last 10 years, intestinal diseases have been intensely researched in Denmark. Research projects have investigated clinical, pathological and microbiological conditions in order to gain knowledge of the most common intestinal diseases in Danish pig production. The overall aim of the projects has been to optimise and reduce antimicrobial use for treatment of intestinal diseases, as this accounts for the majority of antimicrobials used in Danish pig production.

The following section will describe outbreaks of diarrhoea as well as the most common intestinal diseases in pigs. The focus will be on nursery pigs – from weaning to approximately 30 kg bodyweight.

At the end of this section, a new term will be proposed to describe intestinal disease of multiple infectious aetiology.

2.2.1 Description of diarrhoea outbreaks in nursery pigs

Diarrhoea is a common clinical manifestation of intestinal diseases. The primary pathophysiological mechanism of diarrhoea is hypersecretion, malabsorption and inflammation, and depends on the presence or type of infectious agent affecting the intestine (Thomson and Friendship, 2012). The presence of clinical diarrhoea (as assessed by the herd personnel) is one

of the primary reasons for initiating antimicrobial treatment, and it therefore highlights an important aspect of prudent antimicrobial use in the treatment of intestinal diseases (Pedersen et al., 2014a). The 8-week nursery period (from weaning at approximately 4 weeks of age to transfer to the finishing units) is characterised by a high risk of diarrhoea outbreaks. Weaning is one of the most critical periods for pigs, where adaption to solid feed and comingling with other pigs represent key risk factors for intestinal diseases (Nagy and Fekete, 1999).

In the first week after weaning, post-weaning diarrhoea (PWD; also called colibacillosis), is a major disease risk, often with the involvement of ETEC (Frydendahl, 2002; Fairbrother et al., 2005). Prophylactic treatment with in-feed antimicrobials in weaner diets has been commonly used to control PWD, accounting for a large amount of antimicrobial usage worldwide (Page and Gautier, 2012). Following the ban of prophylactic treatment and growth promoters in Danish pig production, in-feed Zinc oxide supplement has become the standard method of controlling PWD (Poulsen, 1995). It is legal to mix 3,000 ppm Zinc oxide in weaner diets for the first 14 days after weaning, and the annual amount of prescribed zinc oxide for prophylactic treatment of PWD was approximately 500 tonnes in 2015 (DANMAP, 2016). This has led to a reduced frequency of PWD outbreaks in Danish pig production, and classical PWD has become less important (Jensen, 2006; Pedersen, 2012).

Once nursery pigs have passed the critical weaning period, the only change occurring from 3 to 8 weeks post weaning is a change in feed from weaner diets to diets based on a high level of soybean. In this period, diarrhoea outbreaks are common and the clinical signs are mainly characterised by diarrhoea and in some cases unthriftiness and increased mortality (Pedersen, 2012). Intestinal pathogens can be detected in many outbreaks of diarrhoea. In a survey from the UK, over 50% of producers reported problems with enteric diseases involving diarrhoea in grower-finisher pigs (Pearce, 1999).

In 20 of 37 Scottish farms with a clinical history of diarrhoea during the growing period (15–40 kg), intestinal pathogens were detected (Chase-Topping et al., 2007).

In some cases of diarrhoea outbreaks in nursery pigs, none of the classical infectious agents can be detected (Smith and Nelson, 1987; Chase-Topping et al., 2007; Pedersen et al., 2010a; Pedersen et al., 2012a). Different terms to describe these outbreaks of non-haemorrhagic diarrhoea with no or a low prevalence of known intestinal agents have been suggested, including non-specific colitis and low-pathogen diarrhoea. Non-specific colitis describes the condition of

diarrhoea outbreaks in pigs from 12-40 kg bodyweight with an absence of pathogens and a lack of any unique pathological lesions (Thomson, 2009; Pedersen et al., 2012a).

Low-pathogen diarrhoea describes diarrhoea outbreaks in nursery pigs 12–63 days post weaning, when low pathogen numbers can be demonstrated in faecal samples (Pedersen et al., 2014a). Under Danish conditions, low-pathogen diarrhoea has been observed in approximately one fifth of the examined diarrhoea outbreaks in nursery pigs (Pedersen et al., 2015).

Since low-pathogen diarrhoea and non-specific colitis are difficult to differentiate from outbreaks caused by infectious agents, there is a risk of unnecessary antimicrobial treatments. Furthermore, the association between clinical diarrhoea and intestinal infections has been proven to be weak. Subclinical *L. intracellularis* infections in individual pigs are well described, and only 50% of pigs with pathological *L. intracellularis* lesions will display diarrhoea (Chouet et al., 2003; Brandt et al., 2010; Pedersen et al., 2010a; Paradis et al., 2012).

2.2.2 Single-pathogen-related intestinal diseases in nursery pigs

Colibacillosis

The causative agent of colibacillosis is enterotoxin-producing β -haemolytic *E. coli*. Clinical signs include diarrhoea, which causes dehydration, emaciation and decreased weight gain in pigs in the first week after weaning (Wills, 2000; Fairbrother et al., 2005). However, infection with ETEC can also be observed in pigs after more than 3 weeks post weaning (Fairbrother and Gyles, 2012; Sato et al., 2016). The clinical signs last up to 1 week, with the pathogenic *E. coli* strains being spread via the faecal–oral route (Fairbrother and Gyles, 2012). The presence of specific serotypes has been correlated with haemolytic *E. coli* causing clinical disease, with the most important being O8, O138, O139, O141, O149 (Frydendahl, 2002). The pathogenic *E. coli* strains are characterised by the production of adhesions and enterotoxins. The most important adhesion factors identified in strains causing clinical diseases are F4 and F18 fimbriae, and the most important enterotoxins are LT, STa and STb (Fairbrother et al., 2005; Chapman et al., 2006; Zhang et al., 2007; Zajacova et al., 2012). Although the presence of pathogenic *E. coli* strains are important in the aetiology of colibacillosis, other non-infectious factors such as suboptimal climate conditions and low feed uptake after weaning contribute to the presence of clinical disease (Madec et al., 2000).

Dysentery

The strongly beta-haemolytic spirochete *B. hyodysenteriae* is the causative agent of swine dysentery, and this major bacterial intestinal pathogen causes high economic losses worldwide

due to increased mortality and reduced growth (Burrough, 2017). Surveillance of *B. hyodysenteriae* in breeding and multiplier herds is part of the Specific Pathogen Free (SPF) system in Danish swine production and all Danish breeder herds are declared PCR-negative. The presence of *B. hyodysenteriae* in Danish production herds is extremely low and therefore largely eradicated so it is no longer considered to be a major bacterial intestinal pathogen in Denmark (Stege et al., 2000; SEGES-VSP, 2015). In Sweden and Denmark, a new atypical strongly beta-haemolytic spirochete named *B. suanatina* has been identified in pig herds suffering from dysentery and diarrhoea (Rasback et al., 2007; Mushtaq et al., 2015). This newly identified spirochete has not been detected in other parts of the world and its significance in causing disease is yet to be established (Hampson, 2012; Burrough, 2017).

Intestinal spirochetosis

Infection by the weakly beta-haemolytic spirochete *B. pilosicoli* causes intestinal spirochetosis and is characterised by mild, usually non-bloody diarrhoea, resulting in poor feed conversion and reduced growth (Trott et al., 1996). Clinical signs usual appear late in the nursery period and can last for 7 to 10 days (Wills, 2000). A wide variation in the severity and prevalence of the disease has been observed between herds, which may be associated with the presence of different strains (Hampson, 2012). Cases of porcine intestinal spirochetosis have been reported from most countries with significant pig production (Hampson, 2012). In Denmark, *B. pilosicoli* was detected in 28 of 75 herds with a history of clinical diarrhoea (Moller et al., 1998). Intestinal spirochetosis caused by *B. pilosicoli* infection is the most economically important disease among those caused by the four weakly haemolytic spirochetes that have been shown to colonise pigs (Hampson, 2012).

Proliferative enteropathy

Infection by the obligate intracellular bacterium *L. intracellularis* can result in different clinical diseases: proliferative haemorrhagic enteropathy, chronic proliferative enteropathy (PE) and subclinical PE (McOrist and Gebhart, 2012). The most common form of *L. intracellularis* infection in grower pigs is chronic PE, clinically characterised by anorexia, reduced weight gain, wasting, diarrhoea and occasionally death (Lawson and Gebhart, 2000). The severity of PE is related to the infection dose. Nursery pigs with an excretion level above 10^7 *L. intracellularis* per gram faeces may have a reduced daily weight gain of 110 g per day over the following 3-week period (Pedersen et al., 2012b). An association between the faecal excretion level of *L. intracellularis* and growth rate has also been reported in grower-finisher pigs, where an increase

of 1 log₁₀ units of *L. intracellularis* doubles the odds of a pig having a low growth rate (Johansen et al., 2013).

Clinical signs are most commonly seen in grower pigs at 6-8 weeks post weaning, but can also be detected in nursery pigs at 3-4 weeks post weaning (Steghe et al., 2004). *L. intracellularis* occurs worldwide and is common in all pig-raising regions (Lawson and Gebhart, 2000). In a Danish study of grower pigs (15-80 kg bodyweight), *L. intracellularis* was detected in 54 of 75 (75%) herds with a history of clinical diarrhoea (Moller et al., 1998).

Salmonellosis

Salmonellosis in pigs occurs worldwide and is mainly caused by *Salmonella choleraesuis* or *Salmonella typhimurium* (Carlson et al., 2012). *Salmonella spp.* is also commonly considered to be a zoonotic agent harboured in pigs without any clinical diseases. *Salmonella choleraesuis* is a rare finding in Denmark, and in 2013 it was only present in three pig herds (Anonymous, 2014). In a Danish study from 1995-1996, *Salmonella typhimurium* was detected in 9% of herds with diarrhoea outbreaks in grower pigs (Moller et al., 1998). This finding could not be confirmed in a study from 2008, where there was no evidence of *Salmonella spp.* in the 20 investigated diarrhoea outbreaks in Danish nursery pigs (Pedersen et al., 2014a). There has been a recent increase in reported cases of salmonellosis in Danish nursery pigs. The cases were characterised by increased mortality, unthrifty pigs, and occasional diarrhoea outbreaks in pigs 2-3 weeks post weaning (Pedersen et al., 2013a).

Porcine Circovirus type 2

Infections with Porcine circovirus type 2 (PCV2) has been associated with enteritis in grower pigs (Opriessnig et al., 2007). Clinical signs and pathological lesions of PCV2-associated enteritis often resemble those associated with *L. intracellularis* infections (Jensen et al., 2006). The aetiological role of PCV2 was also examined in the previously mentioned study of 20 acute diarrhoea outbreaks. There was no significant difference observed in the prevalence and excretion level of PCV2 between pigs with and without diarrhoea, and PCV2 was not considered to be a major intestinal pathogen in nursery pigs in this study (Pedersen et al., 2011a).

Rotavirus

Rotavirus is a major cause of diarrhoea in young pigs (Chatzopoulos et al., 2013). Porcine rotaviruses belong to four serogroups, where group A is the most prevalent throughout the world (Vlasova et al., 2017). In uncomplicated infections with rotavirus, the clinical picture is often 2-3 days of mild diarrhoea with low mortality. Concurrent rotavirus infection with ETEC can

increase the severity of the diarrhoea and result in higher mortality (Chang et al., 2012). In a study of enteric viral infections in five European countries, rotavirus was mainly detected in diarrhoeic pigs (Zhou et al., 2016). Conflicting results of rotavirus as a causative agent of diarrhoea have recently been presented from a study of Danish nursery facilities with diarrhoea outbreaks. Rotavirus was detected in 98% of 40 examined herds, but was demonstrated at equally high prevalence in pigs with or without clinical diarrhoea (Pedersen, 2016).

Porcine Epidemic Diarrhoea

Porcine epidemic diarrhoea is caused by infection with Porcine epidemic diarrhoea virus (PEDV), which is a coronavirus that can infect pigs of all age groups (Saif et al., 2012). In Europe, acute outbreaks of diarrhoea have mainly been reported in nursery pigs (Saif et al., 2012; Lee, 2015).

PEDV is a common pathogen throughout the world, but has never been detected in Denmark (Strandbygaard and Bøther, 2015). After the discovery of a new highly pathogenic strain in the USA and Germany in 2014, a large serological investigation of PEDV in Danish pig herds was conducted, confirming that PEDV is not present in the Danish pig population (SEGES-VSP, 2015; Stadler et al., 2015; Anonymous, 2016).

Transmissible Gastroenteritis

Transmissible gastroenteritis (TGE) is caused by infection with TGE virus, which, like PEDV, is a coronavirus. Clinical signs of watery, yellow to green, malodorous diarrhoea and vomiting occur when TGE virus is introduced to naïve herds. In TGE outbreaks, rapid dehydration following infection leads to high mortality, particularly in piglets. TGE virus can be detected throughout most of the world (Saif et al., 2012) and is a notifiable disease, but has never been detected in Denmark (Anonymous, 1992, 2016).

2.2.3 Causative agents in diarrhoea outbreaks in Danish nursery pigs

Proliferative enteropathy associated with *L. intracellularis* was thought to be the major cause of diarrhoea in weaners and growers in Denmark (Jensen, 2006; Pedersen et al., 2012a). This perception was based on clinical inspections by herd veterinarians and was not thoroughly validated by microbiological and pathological diagnosis. Therefore, a research project was conducted in 2008 to establish the infectious causes of diarrhoea outbreaks in Danish nursery pigs. The study included examinations of 20 diarrhoea outbreaks in commercial nursery herds. On average, outbreaks of acute diarrhoea were observed at 32 days post weaning. The bacterial intestinal pathogens found in this study were ETEC, *L. intracellularis* and *B. pilosicoli* and

mixed infections were common. Intestinal disease associated with a single bacterial pathogen was demonstrated in 40% of the outbreaks, while intestinal disease associated with two or more bacterial pathogens was demonstrated in 60% of the outbreaks (Pedersen et al., 2014a). Mixed infections have also been observed at individual pig level. In a study of 720 diarrhoeic nursery pigs, mixed infections were observed in 19.9% of the pigs (Moller et al., 1998). In another study, one or several of the four pathogens F4/F18 positive *E. coli*, *L. intracellularis* and *B. pilosicoli* were detected by qPCR in 89 (79%) of 113 clinical samples of faecal specimens from pigs with diarrhoea. The samples were taken from 65 different herds, and obtained through routine submissions to the National Veterinary Institute in Copenhagen, Denmark from 2007 and 2008 (Ståhl 2011). In many of the samples, multiple pathogens were detected although not quantified. As a result of these findings, ETEC, *L. intracellularis* and *B. pilosicoli* are now considered to be the major causes of intestinal infections in Danish nursery pigs, and mixed infections are thought to be common.

Similar findings of acute diarrhoea outbreaks caused by mixed infections have been reported from other countries. In Canada, mixed infections by ETEC, *L. intracellularis* and *B. pilosicoli* in nursery pigs have been described (Carpenter and Burlatschenko, 2005). A Swedish study of 13 herds concluded that *L. intracellularis* and *B. pilosicoli* were the main pathogens involved in enteric diseases among Swedish pigs older than 4 weeks post weaning, and that mixed infections were commonly found (Jacobson et al., 2003). In Japan, mixed infections of both bacterial and viral origin were detected in approximately half of 116 faecal samples from diarrhoeic nursery pigs (Katsuda et al., 2006).

2.2.4 Risk factors for intestinal disease

Management and dietary factors can have an influence on both clinical diarrhoea in pigs and on the prevalence of intestinal pathogens.

A high level of non-starch polysaccharides, pelleted feed, stocking density and air quality have all been identified as significant risk factors of non-specific colitis (Chase-Topping et al., 2007; Thomson, 2009). Furthermore, faecal consistency is influenced by the interaction between weaning age and composition of the weaner diet (Callesen et al., 2007).

Pathogen-originated diseases such as colibacillosis are closely associated with dietary components and intestinal barrier functions (Kim et al., 2012). Feeding newly weaned pigs with a high level of dietary protein can result in undigested protein materials in the small intestine.

Undigested protein materials may cause pathogen proliferation and have been identified as one of the most important risk factors in the aetiology of colibacillosis (Jeaurond et al., 2008).

The structure of diets has been shown to be associated with *L. intracellularis* infections. Home-mixed (as opposed to factory processed) diets were associated with a reduced prevalence of *L. intracellularis* and a reduced relative amount of *L. intracellularis* in the total microbiota of the ileum (Stege et al., 2001; Molbak et al., 2008). In the case of intestinal spirochetosis, it has been demonstrated that feeding grower pigs a rice-based diet can reduce the colonisation and excretion of *B. pilosicoli* (Hampson et al., 2000; Lindecrona et al., 2004).

In terms of management factors, animal grouping, buying in replacement stock, mixing animals of different ages and hygiene level have been identified as risk factors for *L. intracellularis* infections (Bane et al., 2001; Chouet et al., 2003; Bae et al., 2013; Resende et al., 2015). Other factors such as weaning age, feed change, significant temperature variation, pig flow and non-slatted floors have been identified as important risk factors that can influence the pressure of intestinal infections (Wathes et al., 1989; McCracken et al., 1995; Mathew et al., 1996; Fairbrother and Gyles, 2012).

2.2.5 Economic impact

Although it has been stated that intestinal diseases are among the most important economic problems affecting pig production, specific studies on the economic impact are lacking (Wills, 2000; Thomson and Friendship, 2012). Assessment of the impact on productivity has mainly focussed on *L. intracellularis* infections, which can lead to reduced growth (Jacobson et al., 2003; Stege et al., 2004; Pedersen et al., 2012c).

Challenge studies in nursery pigs have reported reduced growth rates of 15 g to 230 g per day following inoculation with *L. intracellularis* (Paradis et al., 2012; Collins and Barchia, 2014). In these studies, the level of growth reduction was strongly correlated with the inoculation dose. The total economic loss associated with *L. intracellularis* infections has been estimated at between €0.50 and €11 per affected growing pig in Europe. This is mainly the result of reduced slaughter weight and feed conversion efficiency, increased space utilisation due to low growth, and an increase in morbidity and mortality (McOrist, 2005). Several challenge studies (as recently reviewed by Adewole et al., 2016) have investigated the impact of PWD on growth performance, using pathogenic strains of ETEC. A significant association between ETEC and daily weight gain was reported in three out of six studies included in the review (Adewole et al., 2016).

Reduced performance has also been observed in diarrhoeic pigs with no or a low presence of intestinal pathogens. Pigs suffering from non-specific colitis have poorer feed conversion rate, but the daily weight gain is not affected (Thomson et al., 2006).

2.2.6 Causative relationships between pathogens and intestinal diseases

Intestinal diseases are traditionally viewed as one pathogen – one disease. Porcine epidemic diarrhoea or TGE are examples of pig diseases where the presence of the pathogen in a naïve herd leads to clinical disease.

Another way of viewing intestinal diseases is to take into account the infectious burden of a specific pathogen. In certain intestinal diseases, a pathogen can be present without causing disease before a critical threshold is surpassed. An example is *L. intracellularis* infections, where a correlation between the faecal content of *L. intracellularis* bacteria and the presence of proliferative pathological lesions and reduced growth has been established (Pedersen et al., 2012c; Pedersen et al., 2012d; Collins and Barchia, 2014).

A third approach is to describe the problem of intestinal diseases as a complex, with multiple aspects of pathogens and/or predisposing factors leading to disease. Polymicrobial diseases are defined by clinical and pathological manifestations induced by multiple microorganisms, and are often described as complicated, dual, mixed or secondary infections. The pathogenesis of polymicrobial diseases are influenced by several factors: predisposing factors such as physical and physiological stress in the host, alterations to the mucosa induced by one organism that may favour colonisation by others, synergistic triggering of proinflammatory cytokines, sharing of determinants, and potential distraction of the immune system by one organism that may facilitate colonisation by other organisms (Brogden, 2002). Examples of polymicrobial pig diseases are atrophic rhinitis and porcine respiratory disease complex, where multiple pathogens of bacterial or viral origin can be detected in pigs with clinical disease (Morin et al., 1983; Brockmeier et al., 2002; Chan et al., 2013).

2.3 Porcine intestinal disease complex

Intestinal disease is characterised by the interaction of dietary factors. The gastrointestinal tract contains billions of bacteria as part of the normal digestive system. Different feed compounds and the type of feed influence the microbiota and the potential pathogenic bacteria. As a result, dietary factors can lead to clinical diarrhoea with no presence of pathogens and can influence the

prevalence and severity of pathogens. Furthermore, non-infectious factors (e.g. management) are significant contributors to intestinal disease, either by increasing the spread or transmission of pathogens or by inducing stress resulting in clinical diarrhoea. The classical way of describing intestinal diseases in pigs is single-pathogen-related diseases such as colibacillosis and PE. Yet in many cases, multiple pathogens can be detected in clinical outbreaks of diarrhoea, thereby highlighting the need to define intestinal diseases as polymicrobial, where a combination of concurrent infections with bacterial or viral agents and dietary- and management-related risk factors can amplify the severity of the disease complex (Figure 1).

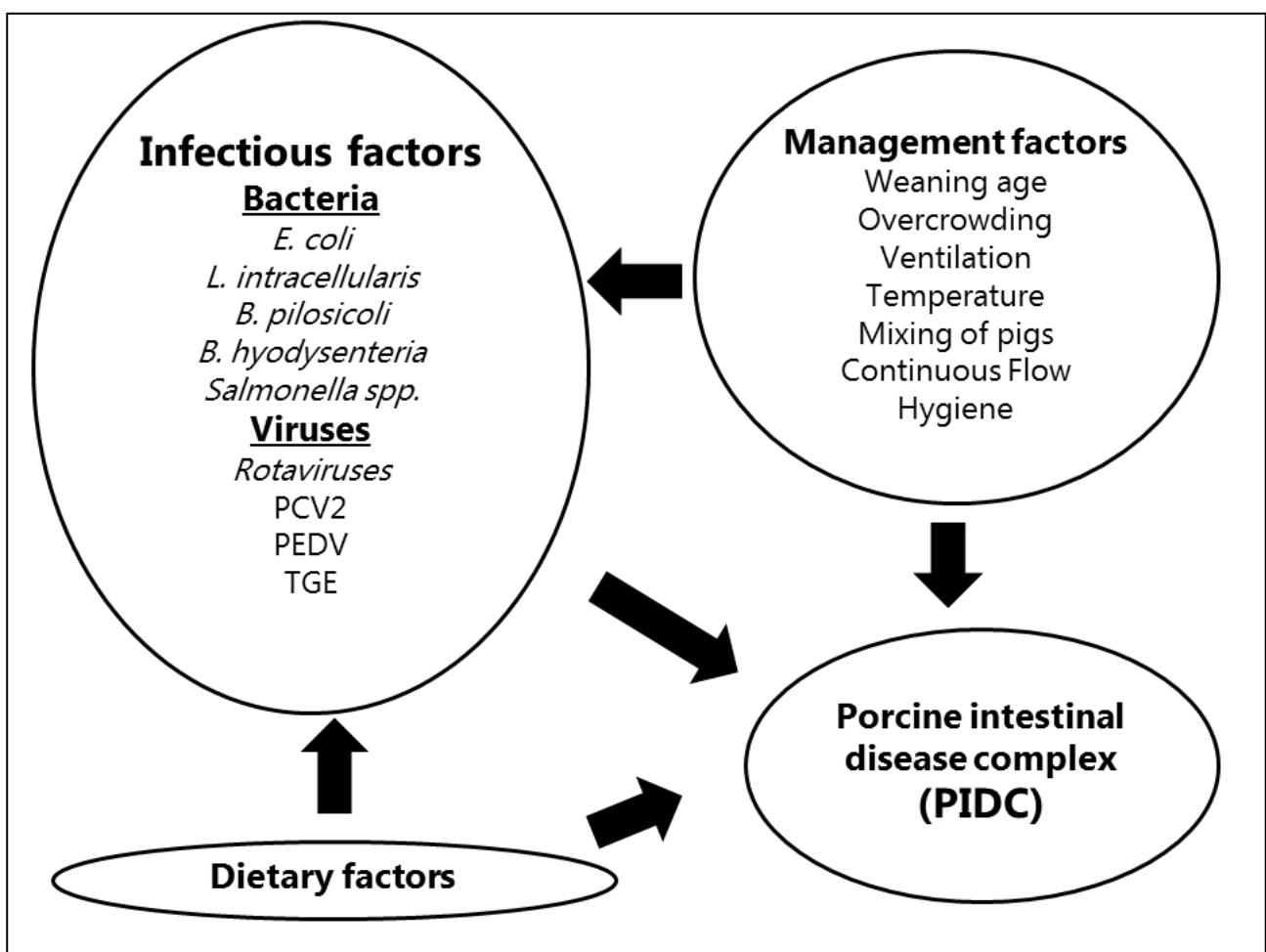


Figure 1 Interaction of infectious, dietary and management factors that result in Porcine Intestinal Disease Complex (PIDC)

The term Porcine Intestinal Disease Complex (PIDC) is suggested to describe diseases of multiple infectious aetiology affecting the small and large intestines and causing clinical disease and failure to gain weight. The clinical manifestation of outbreaks of diarrhoea is a common

feature and factors of both dietary and management origin can influence the condition. The clinical signs of PIDC cannot be differentiated from diarrhoea outbreaks with an absence of known intestinal pathogens. Furthermore, subclinical infections in pigs with pathological intestinal lesions have been observed in nursery pigs with normal faecal consistency.

The composition of infectious agents in cases of PIDC varies between countries and herds. Infectious agents can be simultaneously present at herd, batch and pig level, and the impact of each of the pathogens can be difficult to quantify. When diagnosing causative agents of diarrhoea outbreaks in nursery pigs, quantitative methods with multiple pathogen detection are preferable in order to determine the role of pathogens in PIDC. As previously described, bacterial infections by ETEC, *L. intracellularis* and *B. pilosicoli* are considered to be the main causes of PIDC in Danish pig production, while viral infections are considered of minor importance.

2.4 Diagnosis and treatment of intestinal diseases

Most of the published studies on the diagnosis and treatment of intestinal diseases are based on single-agent infections, in particular colibacillosis and PE (Amezcuca et al., 2002; Guedes et al., 2002; Larsen et al., 2016a; Rhouma et al., 2017). Since the field conditions are often polymicrobial, as described for PIDC, diagnosis and treatment are more complex than the classical approach based on single-agent intestinal disease.

The following section describes the different available methods for diagnosing colibacillosis caused by ETEC, PE caused by *L. intracellularis*, and intestinal spirochetosis caused *B. pilosicoli*. Furthermore, the current method for treating PIDC caused by mixed infections of *L. intracellularis*, *B. pilosicoli* and ETEC in Danish nursery pigs will be described, with a focus on diagnosis, treatment regimens and the selection of antimicrobial agents.

2.4.1 Diagnosis of intestinal diseases

Clinical diagnosis

The first step in a diagnostic workup is to identify clinical signs of intestinal disease based on the evaluation of individual pigs, or on group observations. Clinical signs observed during herd visits can be supported by on-farm records of performance data, mortality and antimicrobial treatments. When diagnosing a herd based on clinical signs, the disease prevalence and sample size should be taken into account. The advantages of a clinical diagnosis are evident – they can

be carried out by the herd veterinarian immediately and control intervention can be put into action without any laboratory costs. The disadvantage is that clinical signs are not always disease-specific, and an accurate diagnosis can be difficult to make.

Common clinical signs of infections caused by ETEC, *L. intracellularis* and *B. pilosicoli* are diarrhoea, emaciation and unthriftiness. Perianal irritation and perianal faecal staining of the individual pig have been identified as predictors of diarrhoea. However, using these clinical signs to estimate the prevalence of diarrhoea in groups of pigs will lead to an underestimation of the true prevalence due to a high level of false negatives (Pedersen et al., 2011b). Counting the diarrhoeic droppings on the pen floor has been suggested as a method to estimate the diarrhoea prevalence, but the association with the true diarrhoea prevalence is also weak (Pedersen et al., 2011c). Due to the similarity of clinical signs, it is therefore necessary to combine the clinical diagnosis with diagnostic testing to make an accurate ante-mortem diagnosis for a group of pigs in order to identify the disease-causing pathogens.

Post-mortem examinations

The next step in the diagnostic workup would be post-mortem examinations, including identification of gross and histopathological lesions in diseased animals. Animals selected for post-mortem examinations must be truly representative of the clinical signs identified by the clinical evaluation of the herd. If tissue or faecal samples are collected for histology and pathogen detection, the selected animals should preferably be in the acute phase of the disease and not have been subjected to antimicrobial treatment. Post-mortem examinations can show the link between clinical findings and pathological lesions. The disadvantage of post-mortem examinations is that pigs must be sacrificed to obtain a diagnosis. Furthermore, interpreting the results from individual pigs in relation to the herd-health status can be challenging.

Pathological lesions in pigs affected by ETEC are generally few and diverse. Gross lesions involve dilatation of the small intestine, with a hyperaemic and sometimes oedematous appearance. Microscopic lesions are generally considered to be absent in ETEC infections (Fairbrother and Gyles, 2012).

Classical pathological lesions of PE consist of proliferation of epithelial cells of intestinal crypts that are most often located in the distal part of the ileum but may also include the caecum (Lawson and Gebhart, 2000). Microscopic lesions of PE are enlarged crypts with immature epithelial cells and an absence of goblet cells (McOrist and Gebhart, 2012).

Gross lesions of intestinal spirochetosis are usually subtle and limited to the caecum and colon (Hampson, 2012). In acute cases, mild signs of congestion and mucosal hyperaemia may be present in the large intestine, and the content can be greenish to yellow (Jensen et al., 2000). In some acute cases, the serosa are thickened by oedema in the large intestine, and the serosa may be thickened by fibrin or fibrous connective tissue in chronic cases (Hampson, 2012). The colonic and caecal mucosa in the affected areas may be congested and thickened by oedema fluid, forming prominent ridges (Thomson et al., 1998). Microscopic lesions caused by intestinal spirochetosis include an increase in the height of the mucosa and the depth of the crypts in the large intestine, and a moderate to marked infiltration of mononuclear cells into the lamina propria (Thomson et al., 1998).

With the knowledge that concurrent infections are common in cases of PIDC, post-mortem examinations can lead to causative infections being overlooked. For instance, if necropsy of diseased animals reveals proliferative lesions in the ileum, a tentative diagnosis would be PE. However, subtle and diverse lesions due to ETEC and *B. pilosicoli* would lead to an inaccurate and incomplete diagnosis, which could result in an improper intervention.

Pathogen identification

To secure an accurate diagnosis, clinical and pathological findings must be supplemented by pathogen detection in sampled tissue or faeces with an appropriate diagnostic test. Immunohistochemistry (IHC) of affected tissue and microbiological culture of faecal samples have historically been standard procedures for intestinal pathogen detection in pigs (Zmudzki et al., 2012). Due to its low cost, fast results and high sensitivity, PCR testing of faecal samples has recently become more popular, making an ante-mortem diagnosis possible (Braun and Chase, 1999). Furthermore, direct multiplex qPCR methods have been developed for testing faecal samples, where the concentration of multiple pathogens can be estimated (Stahl et al., 2011).

Colibacillosis

For many years, bacterial culture followed by either serotyping or detection of toxin and fimbrial genes by PCR has been the routine method for identifying ETEC-associated diarrhoea in nursery pigs. This method is the most simple and effective way to provide a bacteriological diagnosis for the individual pig (Nagy and Fekete, 1999). Haemolytic activity of *E. coli* is related to virulence (Frydendahl, 2002), and high concentrations of *E. coli* in a pure (or nearly pure) culture is generally recognised as being indicative of colibacillosis (Francis, 1999; Pedersen et al., 2014a). An association has been established between the presence of haemolytic *E. coli* in pure/dominant

culture from faeces or jejunum in diarrhoeic pigs and the quantitative level of *E. coli* F18 genes in faeces from nursery pigs. Using a cut-off value of 5.2 log₁₀ F18 CFU/g faeces in pigs with diarrhoea provided a diagnostic sensitivity and specificity of 76.6 and 90.4, respectively (Weber et al., 2017).

Proliferative enteropathy

IHC testing can demonstrate the presence of *L. intracellularis* in classic proliferative lesions related to the disease, and is considered to be the standard method for diagnosing *L. intracellularis*-associated PE (Guedes et al., 2002). This technique is both highly sensitive and specific, but has the disadvantage that pigs must be sacrificed to obtain a diagnosis. Due to the difficulty of routinely culturing *L. intracellularis*, several alternative methods to diagnose PE in live pigs have been developed. Serological testing of serum samples and PCR testing of faecal samples are the most common methods applied in veterinary practice (McOrist and Gebhart, 2012). Serology testing gives information about historical exposure to *L. intracellularis*, and there is a good agreement between seroconversion and the presence of lesions (McOrist and Gebhart, 2012). Since not all *L. intracellularis*-infected animals shed the organism in faeces at detectable amounts, serological testing can be a feasible diagnostic method (Knittel et al., 1998). With the development of assays for testing faecal samples, PCR testing has become a widespread method used to detect *L. intracellularis* (Pedersen et al., 2010b). PCR techniques are superior in detecting *L. intracellularis* in pigs with acute enteritis compared to traditional necropsy and IHC testing or serology (Jacobson, 2003; Jacobson et al., 2004). Danish studies have demonstrated that qualitative PCR testing of faecal samples overestimates the occurrence of pigs with histological PE lesions. Following the development of qPCR methods, the association between the excretion level of *L. intracellularis* and the severity of the disease has been studied. Compared to qualitative PCR, quantitative PCR testing has increased diagnostic performance (Pedersen, 2011). Several studies have documented the association between the excretion level of *L. intracellularis*, pathological lesions and growth performance (Paradis et al., 2012; Pedersen et al., 2012c; Pedersen et al., 2012d; Collins and Barchia, 2014). Furthermore, a threshold excretion level of *L. intracellularis* has been determined by qPCR in pigs with pathological lesions. The optimal diagnostic performance, providing a diagnostic sensitivity of 0.84 and a diagnostic specificity of 0.93, is achieved when a cut-off value of 4.8 log₁₀ *L. intracellularis* bacteria/g faeces is used (Pedersen et al., 2013b).

Intestinal spirochetosis

A definitive diagnosis of intestinal spirochetosis requires the observation of typical colonic lesions and confirmation of infection with *B. pilosicoli* (Stevenson, 1999). Demonstrating *B. pilosicoli* in the colon can be achieved by IHC or by culture. Culture and biochemical testing are very sensitive methods for detecting and identifying *B. pilosicoli* (Rasback et al., 2006). However, this method is time consuming due to the slow-growing nature of intestinal spirochetes (La et al., 2003). In recent years, PCR testing for the presence of *B. pilosicoli* in faecal samples has been developed (La et al., 2003; Nathues et al., 2007; Song and Hampson, 2009; Stahl et al., 2011). Ante-mortem diagnosis of intestinal spirochetosis is therefore now possible, and it has been shown that isolation in faeces correlates with the presence in tissue (Jacobson et al., 2002). As previously mentioned, PCR techniques have been used to detect ETEC, *L. intracellularis* and *B. pilosicoli* in faecal samples. Multiplex PCR testing makes it possible to investigate several intestinal pathogens simultaneously, with a reduction in costs and time spent (Baccaro et al., 2003; Nathues et al., 2007). Quantitative multiplex PCR testing has been developed, where the concentration of several intestinal pathogens in a faecal sample can be determined (Song and Hampson, 2009; Stahl et al., 2011). A correlation has been identified between the excretion level of intestinal pathogens and the presence of disease in the individual pig. Therefore, qPCR analysis is a feasible method when diagnosing intestinal diseases (Pedersen et al., 2012d; Weber et al., 2017). These laboratory techniques make it possible to determine the excretion level of several intestinal pathogens, and therefore to identify pigs suffering from PIDC, those with single-pathogen infections, and diarrhoeic pigs with no intestinal infection.

2.4.2 Diagnosing PIDC by testing pen floor samples

Diseases can be diagnosed based on the assessment of individual animals or herds. The structure of modern pig production, with large groups of individuals reared in the same compartments, makes herd diagnosis relevant when an intervention of vaccination, treatment or prevention is applied to the whole herd or a sub-population (e.g. a whole compartment of nursery pigs). When a herd diagnosis is based on individual pigs, the selected case pigs must be representative of the whole herd/group. Furthermore, extrapolating individual diagnoses to herd diagnosis requires the selection of an appropriate number of case animals and a collection strategy based on the expected prevalence of disease.

Pooled testing of milk, faeces, eggs and animal tissue is increasingly used as a cost-effective alternative to testing of individual samples. The primary advantage of a pooled test over an

individual test is that more individuals can be represented in pooled tests for the same fixed laboratory cost. A disadvantage is the potential decrease in sensitivity compared with individual animal testing (Christensen and Gardner, 2000). In the process of developing an accurate and precise herd diagnosis for intestinal diseases, attention has been directed to examining pooled faecal samples collected from the pen floor. The hypothesis behind this is that a sample collected from the pen floor represents the average excretion levels of intestinal infections in the group of pigs housed in the room/compartment.

In the case of *L. intracellularis*, it has been demonstrated that qPCR testing of pooled faecal samples corresponds to the average excretion level of the individual faecal samples (Pedersen et al., 2014b). Recently, a combination of clinical findings (a count of the average number of diarrhoeic faecal droppings per pen and qPCR testing of pooled faecal samples) has been suggested as a diagnostic method with which to diagnose PIDC (Pedersen et al., 2014a). In this study, diarrhoea outbreaks in nursery pigs were classified as “high pathogenic” when more than 15% of the examined pigs suffered from enteritis, as assessed by pathogen detection. It was established that a “high-pathogenic” diarrhoea outbreak could be diagnosed with a herd-level diagnostic sensitivity of 0.99 and herd-level diagnostic specificity of 0.80, when the average number of diarrhoeic faecal droppings per pen in the room was ≥ 1.5 and the sum of *E. coli* F4 and F18, *L. intracellularis* and *B. pilosicoli* genes determined by qPCR in a pooled faecal sample exceeded 35,000 bacteria/g (Pedersen et al., 2014a).

For many years, sock sampling has been a cheap and effective diagnostic method for the surveillance of *Salmonella spp.* and *Campylobacter spp.* in broiler herds (Skov et al., 1999; Vidal et al., 2013). Taking inspiration from the broiler industry, sock sampling for qPCR testing of *E. coli* F4/F18, *L. intracellularis* and *B. pilosicoli* in pigs has been evaluated. In a study of PIDC outbreaks in 43 herds with nursery pigs, a sock sample was collected by walking in sock-covered boots on the faecally contaminated slatted floor through all affected pens. Furthermore, individual faecal samples were collected from the pen floors and pooled into one pen floor sample. A comparison of qPCR results from the pooled faecal pen floor sample and a sock sample showed excellent agreement. It was concluded that sock sampling, PCR testing and clinical recording can be used as a diagnostic method for PIDC in pigs, and can provide a reliable diagnosis to assist with clinical decisions for treatment and prevention. Consecutive diarrhoea outbreaks in different batches of pigs were examined in 25 herds. The results showed that both the type of diarrhoea and the aetiology changed over time in the majority of herds,

indicating the potential need for frequent diagnostic examinations. As a consequence, sock sampling provides a precise and accurate diagnosis of PIDC at batch level for the examined diarrhoea outbreak, but provides a poor predictor for the following batches of pigs (Pedersen et al., 2015).

2.4.3 Antimicrobial treatments in pigs

Antimicrobials can be administered to treat (*therapy*) or prevent (*prophylaxis*) disease in pigs. Furthermore, administering antimicrobials to clinically healthy pigs during a disease outbreak before clinical signs are visible (*metaphylaxis*) and administering at sub-therapeutic doses in order to improve growth (*growth promoters*) are also used in pig production.

Antimicrobials can be prescribed based on a clinical diagnosis (*empirical therapy*) or a laboratory diagnosis and antimicrobial susceptibility tests (Guardabassi and Kruse, 2008). Empirical treatments are based on clinical experience and therapy is initiated on the basis of an educated presumption in the absence of complete or perfect information. In certain diseases, it is important to take rapid action to combat the infection in order to minimise morbidity, transmission and mortality, instead of waiting for laboratory confirmation of the infectious agent. In human medicine, treatment of community-acquired pneumonia, urinary tract infections and meningitis are examples of empirical treatments (Nau et al., 2015; Fritzenwanker et al., 2016). Examples of situations where empirical antimicrobial treatments may be used in veterinary pig medicine include acute outbreaks of pneumonia caused by *Actinobacillus pleuropneumoniae* and diarrhoea outbreaks, where antimicrobial treatments often are initiated based on a clinical diagnosis (Gottschalk, 2012; Pedersen et al., 2015).

Antimicrobial agents are selected based on the infectious pathogen at a given site of infection, the antibiotic susceptibility of the identified pathogen, knowledge of pharmacodynamics and pharmacokinetics, the risk of side effects, and the cost of treatment and regulations, including withdrawal times (Giguère, 2013). In empiric treatment, the antimicrobial agent is selected based on the results of research identifying the most common pathogens at a given site or in that clinical setting, and on the expected susceptibility of the infectious pathogen (Walker and Giguère, 2006). National guidelines for antimicrobial usage in animals (where the drugs of choice for treating specific diseases are listed based on the expected susceptibility, clinical effect and risk of side effects) can be used by veterinarians as a decision support tool when selecting antimicrobial agents for empirical therapy.

Antimicrobial susceptibility testing

When a pathogen has been identified, testing for antimicrobial susceptibility can guide the clinician to select the most appropriate antimicrobial agent. The goal of a susceptibility test is to evaluate the efficacy of different antimicrobials *in vivo*, based on results achieved by *in vitro* testing of isolates. Antimicrobial susceptibility testing in veterinary medicine is generally performed using one of two methods – disc diffusion or broth dilution – and the results can either be categorical (susceptible, intermediate, resistant) or quantitative, determined by minimum inhibitory concentration (MIC), (Rubin, 2013). Disc diffusion is based on the inhibition of bacterial growth by diffusion of antimicrobial agents. The size of the inhibitory zone is measured and translated to categorical values of resistance. The advantage of the disc diffusion test is that the panel of antimicrobial agents can easily be customised and the method is simple and feasible in veterinary practice. The disadvantage of this method is that information is categorical or semi-quantitative, and it is not possible to determine the precise degree of possible resistance for the analysed pathogen (Jorgensen and Ferraro, 2009).

Broth dilution is performed on plates with different dilutions of antimicrobials, and the MIC value is defined by the lowest concentration where growth is inhibited. Commercially prepared microdilution plates have been developed where the susceptibility against multiple antimicrobial agents can be tested. The advantage of susceptibility testing by microdilution is that information is quantitative and the whole panel of clinically relevant antimicrobial agents can be tested simultaneously. Disadvantages include the cost of preparing microdilution plates, the availability of laboratory facilities, and the lack of customised panels (Jorgensen and Ferraro, 2009).

The results of susceptibility tests are interpreted by either clinical breakpoints or epidemiological cut-offs (Bywater et al., 2006). Clinical breakpoints are used to predict the clinical outcome of treatment with certain antimicrobial agents against a pathogen (Rubin, 2013). Clinical breakpoints are determined by pharmacokinetic studies specific to the species, dosing regimen, type of disease and target pathogen, and relevant clinical breakpoints are published by the Clinical and Laboratory Standards Institute (CLSI) and The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (CLSI, 2013; EUCAST, 2016). Epidemiological cut-offs are used to separate bacterial populations on the basis of MIC distributions, and are mainly used for research studies of resistance. Epidemiological cut-offs are determined by evaluating MIC distribution data of large isolates collection and can be used to split the population into wild type and non-wild type. Epidemiological cut-offs is not based on

pharmacokinetic studies. When interpreting results of susceptibility tests, available clinical breakpoints (rather than epidemiological cut-offs) should be used for guidance in selecting antimicrobial treatments (Schwarz et al., 2010; Rubin, 2013).

Few veterinary-specific clinical breakpoints have been determined, and the classification of veterinary pathogens is often based on clinical breakpoints from human medicine (CLSI, 2013), which can prove to be scientifically problematic (Schwarz et al., 2008).

2.4.4 Antimicrobial treatment of intestinal diseases

Antimicrobial usage patterns in Denmark

Treatment of gastrointestinal diseases and treatment of respiratory diseases are the most common indications for antimicrobial use in modern pig production (Page and Gautier, 2012; De Briyne et al., 2014; European-Commission, 2015). Antimicrobials prescribed for food animals in Denmark are registered in a national database named Vetstat. When antimicrobials are prescribed, information on the type and amount of antimicrobial, administration route, animal species, age group and indication group are registered in the database. There are three age groups for pigs: sows and piglets, nursery pigs and finishers. The indication group relates to organ systems or disease complexes, for example gastrointestinal system, respiratory system, metabolic/digestion/systemic (Steg et al., 2003). In 2015, the total antimicrobial use for pigs in Denmark was 81.5 tonnes of active compounds (DANMAP, 2016). In the period between 2012 and 2015, antimicrobials prescribed for nursery pigs accounted for approximately 80% of the total antimicrobials prescribed for pigs in Denmark, measured in ADD. Of these, approximately 75% were prescribed for gastrointestinal diseases, measured in ADD. In total, 98% of the antimicrobials used for nursery pigs were administered by the oral route, and tetracyclines, macrolides and pleuromutilins accounted for 80% of this oral use (Jensen et al., 2014).

All Vetstat data are based on information about prescribed antimicrobials and not antimicrobial usage. Furthermore, the gastrointestinal system indication group is an unspecific code that includes all types of diagnoses relating to the gastrointestinal tract. Therefore, conclusions based on Vetstat data about antimicrobial usage for certain diagnoses include the assumption that antimicrobials are used by the herd personnel for the same age group and indication group for which they were prescribed.

Drug selection for antimicrobial treatments

Guidelines for the selection of antimicrobial agents are often based on the principle of one pathogen – one disease. In Denmark, evidence-based guidelines covering antimicrobial use in pigs were developed in 2013 (Fødevarestyrelsen, 2013). In these guidelines, recommendations for selecting antimicrobial agents are based on the known susceptibility of the targeted bacteria, pharmacokinetics, and a risk assessment of the impact on human health.

Due to the frequent occurrence of resistance, the recommended procedure for selecting antimicrobial agents in ETEC-related diarrhoea outbreaks should be based on susceptibility testing of ETEC isolates from case pigs, where isolation of *E. coli* by culture is an easy laboratory procedure. Due to large variations in resistance in ETEC isolates, empiric treatment should be based on knowledge of the individual herd and local data on resistance (Burch et al., 2008). Herd medication with apramycin, neomycin or colistin in the water has been reported as a common method of treatment in cases of ETEC outbreaks (Amezcu et al., 2002; Friendship, 2006). In the latest Danish guidelines on antimicrobial use in pigs, recommendations on the type of antimicrobial to be used for treating ETEC-related diarrhoea are based on resistance profiles of clinical isolates of *E. coli* serotype O149 from samples submitted to Danish diagnostic laboratories (DANMAP, 2016). Resistance to colistin has not been detected, and it is listed as a drug of choice for treatment of ETEC (Fødevarestyrelsen, 2013), which was decided before the European Medicines Agency (EMA) decisions regarding colistin (EMA, 2016).

Few studies of resistance profiles of *L. intracellularis* isolates have been published due to the difficulty associated with culturing. The most extensive study of resistance in *L. intracellularis* is based on 10 isolates from North America and Europe – including one Danish isolate (Wattanaphansak et al., 2009). The study concluded that, based on MIC values, the most active antimicrobials (lowest MIC value) were tiamulin, valnemulin and carbadox; chlortetracycline and tylosin were moderately active, and lincomycin were least active. In the Danish guidelines, pleuromutilins are listed as the drug of choice for the treatment of *L. intracellularis* infections, and tetracyclines and macrolides are listed as secondary choices (Burch et al., 2008; Fødevarestyrelsen, 2013). In a survey of Danish swine practitioners, the most common treatment for diarrhoea outbreaks caused by *L. intracellularis* was reported to be pleuromutilins or tetracyclines in water for a duration of 3-7 days (Nielsen, 2010). A large Swedish study of the antimicrobial susceptibility of 324 *B. pilosicoli* isolates showed that the antimicrobials with the lowest MIC value against *B. pilosicoli* were tiamulin, valnemulin, tylvalosin and doxycycline.

Lincomycin showed intermediate activity, whereas tylosin showed low activity in nearly half of all isolates (Pringle et al., 2012). Similar results were recently reported in a study of 24 US isolates (Mirajkar et al., 2016). In both studies, resistance against tiamulin was observed in 10-15% of all *B. pilosicoli* isolates. There have been no reports on resistance in Danish *Brachyspira* spp. isolates. The Danish guidelines recommend that infection with *Brachyspira* spp. (including *B. pilosicoli*) is treated with pleuromutilins (Fødevarestyrelsen, 2013).

Drug selection for PIDC treatments

Given the polymicrobial nature of PIDC, antimicrobial treatment can be complex and problematic, and no guidelines for treatment of polymicrobial disease complexes have been developed. The choice of antimicrobial agent thereby often relies on clinical effect and on a knowledge of the expected susceptibility of the infectious pathogens thought to be involved in the clinical outbreak of diarrhoea. In Denmark, it has been reported that the most common treatment of diarrhoea outbreaks in nursery pigs with unknown infectious pathogens (and thereby a possible polymicrobial involvement) is tetracyclines, pleuromutilins, or macrolides for a duration of up to 5 days (Pedersen et al., 2015).

Drug application method

Antimicrobials can be administered by injection to individual pigs or to herds via feed or drinking water. Feed medication is the most common method of administering antimicrobials in the pig industry worldwide (Burch et al., 2008) and is mostly used for prophylactic treatments and for growth promotion. In Denmark, oral administration of antimicrobials via water is the most common method for treating gastrointestinal diseases in nursery pigs (Jensen et al., 2014), and this method has become more common since the development of soluble antimicrobial formulations and more reliable dosing devices (Burch et al., 2008). Recently, a large field study of *L. intracellularis* treatment with oxytetracylin was conducted to assess the most efficient application method. It was concluded that batch treatment via drinking water with 10 mg oxytetracycline per kg bodyweight for 5 days was the most efficient in reducing both high-level *L. intracellularis* shedding and diarrhoea when compared to the treatment of diarrhoeic pens or individual diarrhoeic pigs with a similar dose of oxytetracycline (Larsen et al., 2016b).

Initiating antimicrobial treatment of PIDC

When initiating antimicrobial treatment, timing is essential in terms of achieving prudent use of antimicrobials in food animals. The overall goal is to initiate antimicrobial treatment only when necessary in groups of diseased pigs, before the negative effect of the infection exceeds the effect of treatment.

All antimicrobials used in Danish pig production are prescribed by certified veterinarians after a herd diagnosis based on clinical signs or laboratory analysis. Antimicrobial treatment based on veterinary instruction is initiated by the herd personnel when clinical signs of the disease for which the antimicrobials are prescribed are present. It has been reported that the presence of clinical diarrhoea, as assessed by the herd personnel, is a primary reason for antimicrobial treatment of PIDC in Denmark (Pedersen et al., 2014a). In that study, 84% of the herds where oral antibiotics had been prescribed used some clinical inspection criteria to determine the time point at which antimicrobial treatment for intestinal diseases should be initiated in a batch of pigs. In the remaining 16% of the herds, antimicrobials were used systematically on a fixed day post-weaning, indicating a more prophylactic application of antimicrobials (Pedersen et al., 2015).

3 Study objectives and hypothesis

Based on information in the introduction and background chapters, four hypotheses were formulated:

1. New objective methods to identify intestinal infections in nursery pigs are necessary to ensure the prudent use of antimicrobials
2. The efficacy of batch treatment depends on the correct timing of the treatment and the choice of antimicrobial
3. Testing of pen floor samples for intestinal pathogens may improve the prudent use of antimicrobials for batch treatment of intestinal infections in nursery pigs
4. Intestinal infections in batches of nursery pigs are often of mixed aetiology

To test the hypotheses of this thesis, three studies were carried out in commercial Danish herds with nursery pigs, using commercially available test methods:

Study 1:

A prevalence study evaluating whether clinical assessment of groups of nursery pigs and visual assessment of diarrhoeic faecal droppings on the pen floor can be used as a method to identify diarrhoeic nursery pigs with intestinal infections.

The specific objective of this study was to assess the prevalence of intestinal infections and diarrhoea in clinically healthy nursery pigs.

Study 2:

A field trial assessing the efficacy of antimicrobial treatment regimens for mixed intestinal infections in nursery pigs.

The specific objectives of this study were to:

1. Assess the effect on ADG of four 5-day treatment strategies initiated at fixed time points – 14, 21, or 28 days after weaning, or at the clinical outbreak of diarrhoea
2. Compare the effect of treatment with doxycycline and tylosin on diarrhoea prevalence, pathogenic bacterial load, and ADG

3. Evaluate PCR testing of faecal pen floor samples as a diagnostic tool for determining the optimal treatment effect

Study 3:

A comparative study of three diagnostic methods for detecting ETEC-positive diarrhoeic pigs and antimicrobial susceptibility testing

The specific objectives of this study were to:

1. Investigate whether bacterial culture and qPCR testing of faecal pen floor samples could be used as a diagnostic approach for identifying ETEC in groups of nursery pigs with clinical diarrhoea
2. Evaluate whether ETEC isolates from pen floor samples can be used for resistance profiling in groups of nursery pigs with clinical diarrhoea

4 Materials & Methods



4.1 Study designs

Several study designs were used in this project due to the different study objectives.

An observational study with a cross-sectional design was chosen to address the outcome of interest for diarrhoea prevalence in Study 1. The target population was weaned pigs at an age where acute outbreaks of diarrhoea typically occur. A previous study observed that, on average, outbreaks of acute diarrhoea occur at 32 days post weaning (Pedersen et al., 2014a). Therefore, a study population of batches of pigs 10–66 days after weaning, in herds experiencing outbreaks of acute diarrhoea was chosen. To establish the association between clinical diarrhoea and the prevalence of intestinal pathogens, a nested case-control study of selected pigs was included.

To evaluate the efficacy of treatment strategies and type of antimicrobial in Study 2, an experimental/controlled non-blinded field trial was conducted. To address objectives 1 and 2, a trial with a 2x4 factorial design with two antimicrobial agents and four treatment strategies was chosen, with group allocation by cluster randomisation.

To address objective 3, a nested case-control design was chosen, including pens selected for strategy 1. The included pens were classified as bacteria-positive or –negative, based on the qPCR test results from pen floor samples collected on the day of treatment initiation. This classification was made retrospectively because laboratory testing was necessary. The outcome was ADG, and the primary explanatory variable was the qPCR test result on the day of treatment.

To address the objectives in Study 3, a cross-sectional design was selected with the same target population as in Study 2. A comparative study of three diagnostic methods for detecting ETEC was carried out. Furthermore, the resistance profiles of *E. coli* isolates were compared.

4.2 Description of datasets

Table 4 provides an overview of the three datasets with a description of the design, sample size, and study objectives included in the papers and the thesis. A detailed description of each is given in the respective papers. The following sections give a summary of the datasets.

Study 1

This study was planned and conducted in autumn 2011 by DVM Lise-Lotte Pedersen and DVM Alex Stricker Jacobsen as part of their Master's thesis. Data analysis was performed by the author, and a description of the data collection is given in Paper I.

Data were collected from 16 commercial pig herds that had previously been visited in connection with a study on diarrhoea in nursery pigs (Pedersen et al., 2014a). The herds were characterised by the regular therapeutic use of oral antimicrobials for treatment of intestinal diseases in nursery pigs. The main purpose of this study was to evaluate the procedure performed by herd personnel when initiating antimicrobial treatment for intestinal diseases by assessing diarrhoea prevalence and diarrhoeic faecal droppings on the pen floor. The diarrhoea prevalence and intestinal infections in batches of nursery pigs that were assessed to be healthy were therefore investigated. The dataset included data from clinical examinations and the diarrhoea consistency of 200 randomly selected pigs between 10 and 66 days post weaning in batches that had not been subjected to antimicrobial treatment from 16 herds. Also included were data from qPCR results for *E. coli* F4 and F18, *L. intracellularis* and *B. pilosicoli* derived from 256 randomly selected faecal samples, from a total of 2,866 pigs.

Study 2

The field trial was planned, conducted and analysed by the author. Data were collected between January 2014 and October 2014, and a description of the data collection is given in Paper II.

The study was carried out in herds with weaned pigs housed in nursery facilities for approximately 8 weeks. Herds were selected from pig producers serviced by two veterinary practices in the eastern part of Denmark. Herds with Oedema disease, *Brachyspira hyodysenteriae*, salmonellosis, atrophic rhinitis, and other acute diseases, as well as herds with a vaccination programme against *L. intracellularis* were excluded. Inclusion criteria were: the occurrence of high-pathogenic diarrhoea outbreaks (defined as an outbreak with ≥ 1.5 diarrhoeic faecal droppings per pen and faecal pool samples containing $\geq 35,000$ bacteria per gram faeces, calculated as the sum of *E. coli* F4 and F18, *L. intracellularis* and *B. pilosicoli* per gram faeces tested by qPCR), SPF status, fulfilment of the Danish nutrient standards in feed recipes, use of Zinc oxide 3,000 ppm in the weaner diet for the first 14 days after weaning, two climate pens with partly slatted floors, and all-in all-out batch production in sectioned compartments.

Three study herds were selected, and the distribution of selected pens after randomisation is shown in Table 1.

Table 1 Distribution of pens by antimicrobial and strategy group after randomisation in Study 2

No. of pens in each combination														
	Strategy	S1			S2			S3			S4			Total
	Herd	A	B	C	A	B	C	A	B	C	A	B	C	
Antimicrobial	Doxycycline	4	4	2	4	4	2	5	4	2	4	3	1	39
	Tylosin	4	4	2	4	4	2	5	4	2	4	3	1	39
Total		18			18			22			16			78

The selected double pens were allocated to treatment strategy and antimicrobial groups. The four strategies tested were: strategy 1 (S1): 5 days of antimicrobial treatment initiated 14 days after weaning; strategy 2 (S2): 5 days of antimicrobial treatment initiated 21 days after weaning, or at an earlier time point if there was an outbreak of clinical diarrhoea; strategy 3 (S3): 5 days of antimicrobial treatment initiated 28 days after weaning, or at an earlier time point if there was an outbreak of clinical diarrhoea; strategy 4 (S4): 5 days of antimicrobial treatment only initiated in response to an outbreak of clinical diarrhoea. An outbreak of clinical diarrhoea was defined when either $\geq 50\%$ of pigs were diagnosed with diarrhoea, or $>50\%$ of pigs were treated individually for intestinal disease. Two different active compounds (doxycycline/tylosin) were used in parallel throughout the study. The selected active compounds were based on their relevance to Danish pig production.

The dataset included the weight of individual pigs measured on the first day of the study and on the last day of the study. Furthermore, qPCR results for *E. coli* F4 and F18, *L. intracellularis* and *B. pilosicoli* derived from pen floor samples were included in the dataset, along with data from 10 batches with 78 pens and 1,047 pigs. In the original study plan, two additional batches from Herd C should have been included in the study, but were excluded due to a fire in the herd facilities.

Study 3

Study 3 was planned, conducted and analysed by the author. This study was conducted in the same herds and at the same time as Study 2. A total of 93 pigs housed in 31 pens were sampled in this study.

The dataset included registrations of haemolytic activity, toxin and fimbriae gene detection and MIC values for 13 different antimicrobial agents of sampled *E. coli* isolates. In addition, qPCR results for *E. coli* F4 and F18, *L. intracellularis* and *B. pilosicoli* derived from pen floor samples were included in the dataset, along with 208 *E. coli* isolates from rectal faecal samples, 172 isolates from pen floor samples and results from qPCR tests of 31 pen floor samples.

4.3 Sample size considerations

Sample size calculations were performed prior to conducting Study 1 and 2. In Study 3, sampling was performed when the predefined inclusion criteria were fulfilled, and no sample size calculations were performed.

Study 1

In order to calculate the required sample size to estimate a proportion, the following formula was used:

$$n = \frac{(Z_{1-\alpha/2})^2 \times p(1-p)}{L^2} \quad \text{Eq. 1}$$

Where n = sample size, $\alpha = 0.05$ (95% level of confidence), p = estimated prevalence of diarrhoea, and L = accepted error.

The sample size within the herd was calculated with a 4% accepted error. Based on assumptions about the average size of weaning facilities for 4,000 weaners, and a prevalence of diarrhoea estimated at 10% based on best guess, a sample size of 206 pigs from each herd was required. When taking the assumptions into consideration, a sample size of 200 was considered to be sufficient.

In order to estimate the optimal sample size for determining the prevalence of intestinal infections in pigs with and without diarrhoea, an estimated prevalence of $p=0.5$ was chosen.

A sample size of 128 pigs with and without diarrhoea was required to determine the prevalence with an allowable error of ± 0.10 and a confidence level of 95%.

Study 2

Preliminary sample size calculations were performed using formulae for differences in the mean between two groups. The groups were allocated by cluster-randomisation at pen level. The effect of clustering in relation to ADG was taken into account during sample size calculations, as previously described (Dohoo, 2009).

The study was designed to detect a difference of 50 g in mean daily weight gain between pigs in different pens.

Firstly, the sample size for a non-clustered design was calculated using the formula for a two-sided test of difference in means:

$$n = \frac{2 \times (Z_{\beta} + Z_{1-\alpha/2})^2 \times \sigma^2}{(d)^2} \quad \text{Eq. 2}$$

The ADG was estimated at 450 g, and the standard deviation (σ) was 113 g. To achieve a power of 80%, the required sample size in each group was 81 pigs.

The number of pigs required in each group with clustering was calculated using the following formula (Dohoo, 2009):

$$n' = (n(1 + \rho(m-1)))/m \quad \text{Eq. 3}$$

Where n' = new sample size, n = original sample size, ρ = intraclass correlation coefficient (ICC), m = pigs per pen

With clustering, ICC was calculated using the following formula (Ukoumunne et al., 2002):

$$\rho = \sigma^2_B / (\sigma^2_B + \sigma^2_W) \quad \text{Eq. 4}$$

Where, ρ = ICC, σ_B = standard deviation between pens set at 35 g, σ_W = standard deviation between pigs within pens set at 113 g, resulting in a calculated ICC of 0.088.

The number of selected pigs in the study pens was fixed at 15 pigs.

Using Equation 3 with the following values: n (original sample size) = 81 pigs, ρ (ICC) = 0.088, m (pigs per pen) = 15 pigs, the new sample size (n') was calculated to be 180 pigs per group.

The number of pens needed per group was therefore $180/15 = 12$ pens.

A total of eight different factorial study groups (two antimicrobial groups, four strategy groups) were included in the design, giving a total of $8 \times 12 = 96$ pens needed in the study.

Given that the study was to be performed in 12 batches, eight pens were selected in each batch.

The 12 batches were to be distributed among three different herds, as this was the minimum number necessary to use herd as a random effect during the statistical analysis.

4.4 Randomisation

The method of randomisation used in Studies 1 and 2 was generally systematic random sampling, as described in the materials and methods section of Papers I and II. In the following section, the practical approach to selection and randomisation are elaborated further.

Study 1

Upon arrival at the herd, the pens with pigs that fulfilled the inclusion criteria described in Paper I were identified. Starting with the pen with the youngest pigs, each included pen was given a unique number. The sampling interval j was calculated as the number of included pens/20, and every j^{th} pen was selected. If there were less than 20 pens included then all pens were selected.

The first pen was randomly selected among the first j samples and afterwards the pens were chosen by systematic sampling. The same procedure was used to select the pigs within the pens. Using two colours of marker spray, every j^{th} pig was marked for examination with sampling interval $j = \text{pigs}/10$. If there were less than 10 pigs in the pen, all pigs were examined. The included pigs were then given a clinical examination by inspection, and a faecal sample was taken from the rectum. Empty pigs were excluded from the project. In each herd, 200 rectal samples were collected, from which 8 diarrhoeic samples were selected by systematic random sample. The sampling interval J was calculated as the number of diarrhoeic samples/8. To minimise bias, eight non-diarrhoeic samples were randomly selected from the same pens as the diarrhoeic samples, and paired at pen level.

Study 2

Each batch included in the study was included 14 days after weaning. Upon arrival at the herd, the batch with pigs 14 days post weaning was identified. In order to control for the confounding effect of weight/size at weaning in relation to ADG, pens with small-sized pigs and pens with large-sized pigs were excluded before randomisation. Treatment strategy and antimicrobial groups were allocated by drawing lots. Within the selected batch, four double pens that shared the same feeder were randomly selected. Four lottery tickets were prepared with numbers 1 to 4, corresponding to treatment strategy 1-4; the remainder of the tickets were blank. After shaking the bag, pens were allocated by drawing lots starting with the double pen on the left side nearest the entrance door. After the allocation of treatment strategy, the two pens sharing a feeder were allocated to an antimicrobial group. This was done by drawing two pieces of paper labelled 'tylosin' or 'doxycycline' from a bag, starting with the pen located to the left of the feeder. Following pen allocation, 15 pigs from each of the selected pens were chosen by systematic random sampling as indicator pigs. The sampling interval was: $j = \text{pigs per pen}/15$. If there were fewer than 15 pigs in the selected pen, all pigs were selected. All pigs were individually released from behind a large board in the corner of the pen, and every j^{th} pig was marked by spray paint.

4.5 Data collection

Clinical recordings

Clinical recordings were only performed in Study 1. The diarrhoeic faecal droppings on the floor of each included pen were counted. To classify a faecal dropping as diarrhoeic, a categorical faecal consistency scale of 1-4 was used, where 3 and 4 (loose or watery droppings) were classified as diarrhoea (Pedersen and Toft, 2011). The selected pigs were restrained and subjected to a clinical examination by visual inspection. It was possible for each pig to have more than one clinical registration. The pigs were given a clinical score from a predetermined list of clinical signs.

Table 2 List of clinical registrations used in Study 1 for the examination of selected pigs

Clinical sign	Score
Body condition	Normal/abnormal
Contours of the pelvis	1/0
Contours of spinal processes	1/0
Hollow lumbar region	1/0
Abdominal distension	1/0
Long hair coat	1/0
Anaemic	1/0
Faecal staining of hindparts	1/0
Hyperaemic anal region	1/0
Umbilical hernia	1/0
Inguinal hernia	1/0
Unthrifty	1/0
Lameness	1/0
Skin disease	1/0
Dyspnoea	1/0
Cough	1/0
Other	1/0

Measurement of weight

Body weight was used in Study 2 to calculate the ADG. All pigs were weighed at the start of the trial (14 days after weaning) and at the end of the trial (35 days after weaning), using a scale (“Bjerringbrovægt1298GE”) with a precision of 100 g.

Faecal sampling procedure

Study 1

A faecal sample was obtained from each selected pig in the 16 study herds (either by collecting freshly deposited faeces or by digital rectal manipulation using a glove) and stored in sealed plastic containers.

Study 2

An overview of the sampling procedure in Study 2 is shown in Figure 2. Each selected batch was examined four times, once per week. Faecal examination was carried out by scoring faecal samples collected from each pig by digital rectal manipulation using a gloved hand at the start of the study, on the day the pigs were treated (according to the allocated treatment strategy) and at the end of the study. The faecal samples were scored by one observer using the previously mentioned faecal consistency scale with four categories, in which scores 1 and 2 represent normal faeces and scores 3 and 4 represent diarrhoea. Pen floor samples were collected weekly from each of the selected pens and stored in plastic containers, giving a total of four pen floor samples from each pen.

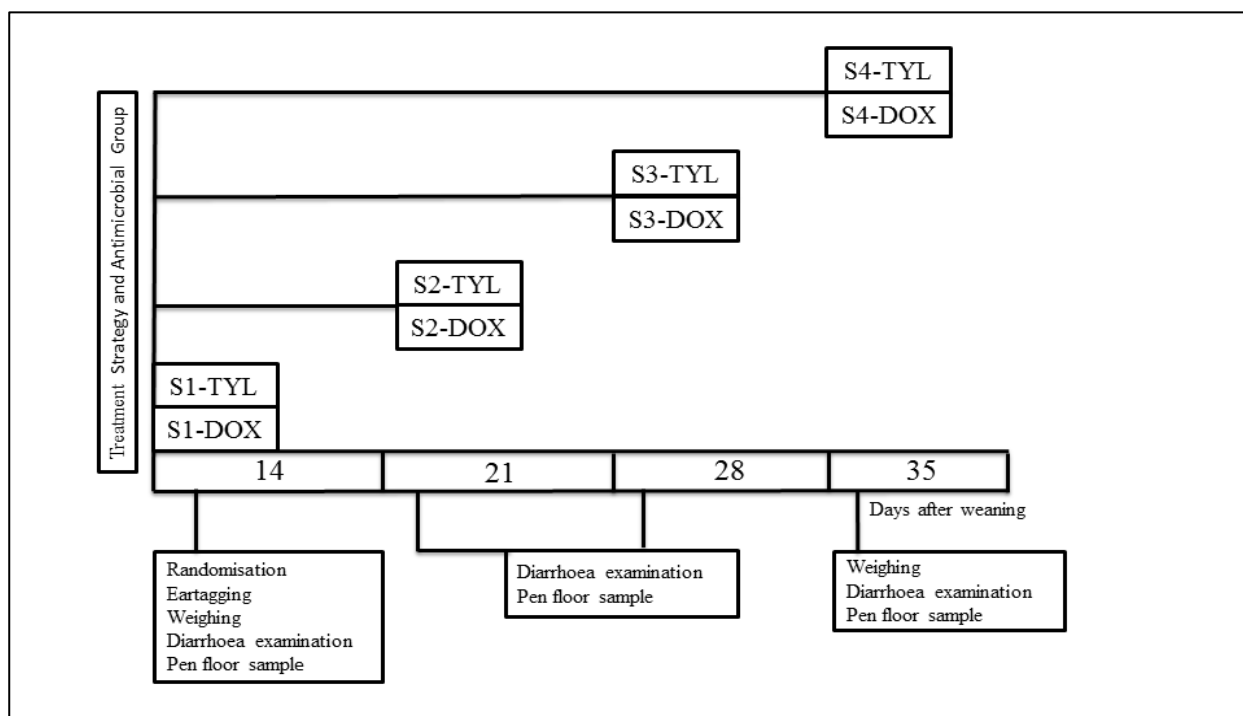


Figure 2 Illustration of sampling and treatment procedure in one batch in Study 2

Study 3

An overview of the sampling procedure for Study 3 is shown in Figure 3. Sampling for Study 3 was performed in pens that were included in Study 2. All pens where antimicrobial treatment had been initiated at pen level were excluded for this study. The inclusion criterion was pens where a clinical diarrhoea prevalence of >25% was observed among the 15 selected pigs. When pens with this diarrhoea prevalence were identified, rectal samples from three diarrhoeic pigs and a pen floor sample were collected. The pen floor sample (approx. 10 g of faeces) was divided into two subsamples. The pig samples and one of the pen floor subsamples were then shipped to the Laboratory for Pig Diseases for bacteriology. The second subsample from the pen floor was shipped to the Danish Veterinary Institute for qPCR analysis.

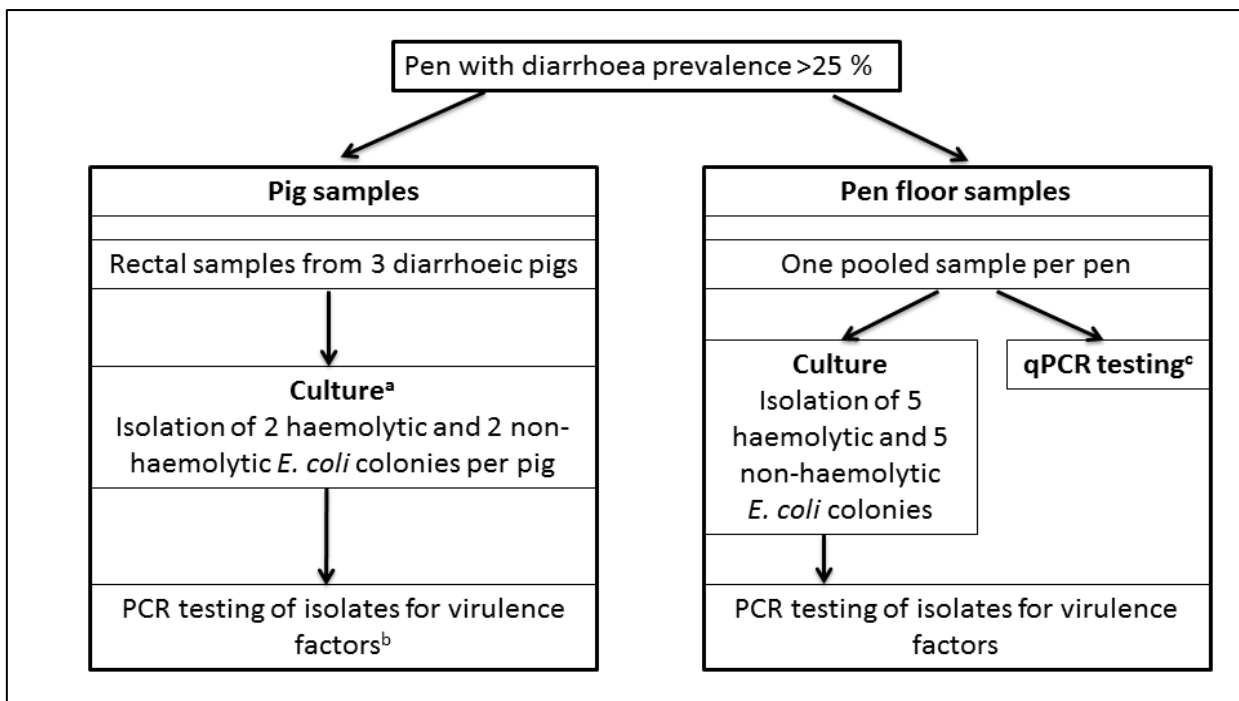


Figure 3 Overview of sample procedure at pen level in Study 3

^aColonies surrounded by a zone of lysis after overnight growth at 37°C on blood agar were classified as haemolytic, ^bDetection of virulence factor genes F4, F5, F6, F18, F41, STa, STb, LT and VT2e by PCR, ^cQuantitative PCR for *E. coli* fimbrial genes F4 and F18, *L. intracellularis* and *B. pilosicoli*.

Where present, two haemolytic colonies surrounded by a clear zone of lysis, and two non-haemolytic colonies were isolated from the pig samples, and five haemolytic colonies and five non-haemolytic colonies were subcultured from the pen floor samples. In cases of pure culture, only haemolytic or non-haemolytic colonies were isolated.

4.6 Antimicrobial treatments

All antimicrobial treatments used in Study 2 for pen-level treatment were dissolved in water administered via troughs positioned in the middle of the pen. The amount of antimicrobial required for a daily dose was determined by multiplying the average weight of the indicator pigs by the total number of pigs in the pen. When the daily dose was determined, five plastic cups containing the correct amount of antimicrobial were prepared. The daily dose was divided equally over two daily administrations in order to increase the chances of uniform dosing. The first treatment was initiated by Nicolai Weber and the following treatments were performed by the herd personnel. All pen-level treatments were initiated according to the treatment protocol. To ensure animal welfare, herd personnel were allowed to treat individual pigs with clear clinical signs of intestinal or other diseases. Individual treatment of intestinal disease was initiated in approximately 10% of the pigs, and the majority of individual treatments were initiated before the pen treatment.

4.7 Laboratory analysis

All laboratory analysis used in the project is commercially available and routinely used in Danish veterinary pig practice. Bacteriology and susceptibility testing were performed at the Laboratory for Pig Diseases, Kjellerup, Denmark. All PCR and qPCR analyses were performed at the Danish Veterinary Institute, DTU, Frederiksberg, Denmark. A thorough description of the laboratory analysis used in Studies 1, 2 and 3 are given in the papers included in this thesis. The main methods are described in the following section.

Bacteriology & Virulence determination

Faecal samples were aerobically cultured for *E. coli* following standard protocols by parallel culturing on Drigalski (an in-house selective and indicative medium for coliforms) and blood agar plates (Columbia agar (Oxoid) supplemented with 5% calf blood). The plates were incubated for 24 hours at 37°C. The isolates were then shipped to the Danish Veterinary Institute and analysed by real-time PCR for the detection of virulence factor genes F4, F5, F6, F18, F41, STa, STb, LT and VT2e, as previously described by Frydendahl et al. (2001) with the exception that PCR cycling was performed on a Rotor-Gene Q (QIAGEN) PCR machine (Frydendahl et al., 2001); Papers III & IV).

Susceptibility testing

Susceptibility testing was carried out for all *E. coli* isolates following the standard protocol of the Laboratory for Pig Diseases. Minimum inhibitory concentrations (MIC) of the isolates were determined by the broth microdilution method in 96-well microtitre plates, using the Sensititre system (Thermo Fisher Scientific, Waltham, Massachusetts, USA), as described in the Clinical and Laboratory Standards Institute (CLSI) standards manual (CLSI, 2013). *E. coli* reference strain ATCC 25922 was used as a control organism. The plates were incubated for 20 hours at 37°C in an aerobic atmosphere. The MIC was defined as the lowest concentration producing no visible growth. The antimicrobial concentrations and clinical breakpoints of 13 antimicrobials used in the susceptibility test are shown in Table 3.

Table 3 Antimicrobial concentration ranges and resistance breakpoints used in Study 3 for susceptibility testing of *E. coli* isolated from faecal samples from weaned pigs and pen floors

Antimicrobial agent	Concentration used (µg/ml)	Clinical breakpoint (µg/ml)
Ampicillin	1 - 32	≥32 ^a
Amoxicillin/clavulanic acid	2/1 - 32/16	≥32/16 ^a
Trimethoprim	1 - 32	≥16 ^b
Sulphamethoxazole	64 - 1024	≥512 ^a
Gentamicin	0.5 - 32	≥16 ^a
Apramycin	4 - 32	≥16 ^b
Streptomycin	8 - 128	≥32 ^b
Spectinomycin	16 - 256	≥128 ^b
Neomycin	2 - 32	≥16 ^b
Ciprofloxacin	0.015 - 4	≥4 ^c
Ceftiofur	0.5 - 8	≥8 ^d
Tetracycline	2 - 32	≥16 ^a
Colistin	1 - 16	≥16 ^b

^aCLSI-approved breakpoints based on human data. ^bBreakpoints routinely used by the Laboratory of Swine Diseases, Kjellerup, Denmark, and by the Danish Veterinary Institute, Frederiksberg, Denmark. ^cCLSI-approved breakpoint for Enrofloxacin based on dog data and assumed to be representative of Ciprofloxacin. ^dCLSI-approved breakpoint based on cattle data.

The antimicrobials used in the susceptibility test were a standard panel used by the Laboratory for Pig Diseases, comprising clinically relevant antimicrobial agents for the treatment of porcine *E. coli* infections. Where available, CLSI breakpoints and those routinely used by the Laboratory of Swine Diseases, Kjellerup, were used to interpret MIC values (Paper IV).

qPCR testing of pen floor samples

All pen floor samples were analysed by the Danish Veterinary Institute. Approximately 1 g of the sample was homogenised in a stomacher for 1 minute with phosphate buffered saline (PBS) to obtain a 10% (w/v) faecal suspension. An aliquot of the suspension was transferred to a 2 ml microfuge tube and stored in a freezer at -20°C until DNA extraction. DNA was stored in a -20°C freezer until the *E. coli* F4/F18, *B. pilosicoli*, and *L. intracellularis* content could be quantified by qPCR, as previously described by (Stahl et al., 2011), with the exception that standard curves for quantification were prepared from DNA extracted from faeces spiked with 10-fold dilution series of the corresponding pathogen, using the same extraction procedure as for the faecal specimens (Pedersen et al., 2012c). Detection limits per gram faeces were: 5.7×10^4 colony-forming units (CFU) for *E. coli* F4; 1.5×10^3 CFU for *E. coli* F18; 2×10^3 bacteria for *L. intracellularis* and *B. pilosicoli*. Linear ranges were: 5.7×10^9 - 5.7×10^5 CFU/g faeces for *E. coli* F4; 1.5×10^{10} - 1.5×10^5 CFU/g faeces for *E. coli* F18; 2×10^8 - 2×10^4 bacteria/g faeces for *L. intracellularis*; 2×10^8 - 2×10^4 bacteria/g faeces for *B. pilosicoli* (Papers I, II & III).

4.8 Data management and statistical analysis

Data collected at herd visits were registered in pre-prepared record forms. The data from the original record forms were entered twice into an Excel 2010 spreadsheet and proofread for missing or inconsistent values. All data from laboratory analysis was received in Excel spreadsheet format and merged with the spreadsheet containing herd data into one master file. After all registrations from herd visits and laboratory analysis had been entered into the master file, data were imported to the statistical program R for statistical analysis (R-Core-Team, 2014). An overview of the statistical method used for each dataset for the different study objectives is presented in Table 5. A detailed description of the statistical analysis is given in the papers. Data from all three studies were clustered and mixed models were therefore used for statistical analysis. In Papers I and II, mixed models with weight gain as outcome were used to account for random effects of herd, batch and pen variations. In Paper IV, mixed models with ETEC status as outcome were used to account for random effects of Batch, Pen and Sample variations. To estimate the effect of antimicrobial agent on the excretion of pathogenic bacteria (Paper II), a Kruskal-Wallis rank sum test was used because the data on excretion levels were not normally distributed.

Table 4 Overview of dataset included in the thesis

Dataset	Objectives	Study design and sample size	Observational unit	Recordings
Study 1	Determine the within-herd prevalence of diarrhoea in nursery pigs in batches of pigs with no signs of intestinal disease	Cross-sectional study, 16 herds, 62 batches 62 pens, 2,866 pigs 256 faecal samples analysed by qPCR	Pig	Faecal score, clinical signs, load of <i>E. coli</i> F4/F18, <i>L. intracellularis</i> and <i>B. pilosicoli</i>
	Determine the prevalence of intestinal pathogens in pigs with and without diarrhoea		Pen	Diarrhoeic faeces on pen floor
			Batch	Days after weaning
Study 2	Assess the effect of four 5-day treatment strategies on ADG	Non-blinded randomised, controlled clinical trial with a 2×4 factorial design, 3 herds, 10 batches, 78 pens, 1,047 pigs, 180 faecal pen floor samples analysed by qPCR	Pig	Live weight, faecal score, mortality
	Compare the effect of treatment with doxycycline or tylosin on diarrhoea prevalence, pathogenic bacterial load and ADG		Pen	Pigs per pen, load of <i>E. coli</i> F4/F18, <i>L. intracellularis</i> and <i>B. pilosicoli</i> , antimicrobial treatments
	Evaluate PCR testing of faecal pen floor samples as a diagnostic tool for determining the optimal time of treatment		Batch	Days after weaning
Study 3	Evaluate whether bacterial culture or qPCR testing of faecal pen floor samples can identify ETEC in groups of weaned pigs with clinical diarrhoea	Cross-sectional study. 3 herds, 31 pens 93 pigs, 208 <i>E. coli</i> isolates from rectal faecal samples and 172 isolates from pen floor samples	Isolate	Haemolytic activity, detected virulence factors, MIC values
	Evaluate whether ETEC isolates from pen floor samples can be used for resistance profiling		Pig sample	Count of <i>E. coli</i> isolated per sample, presence of haemolytic/non-haemolytic <i>E. coli</i>
			Pen floor sample	Count of <i>E. coli</i> isolated, presence of haemolytic/non-haemolytic <i>E. coli</i> , load of <i>E. coli</i> F4/F18, <i>L. intracellularis</i> and <i>B. pilosicoli</i>

Table 5 Overview of statistical analyses performed for each dataset and specific study objectives

Dataset	Study objectives	Response variable	Observational unit	Statistical method	Paper
Study 1	Determine the within-herd prevalence of diarrhoea	Diarrhoea prevalence	Herd	Chi square test	I
	Estimate the association between age and diarrhoea status	Diarrhoea status	Pig	Generalised linear mixed model	
	Determine the prevalence of <i>E. coli</i> F4 and F18, <i>L. intracellularis</i> and <i>B. pilosicoli</i> by qPCR in pigs with and without diarrhoea	Mean qPCR results	Pig	Student's t-test	
Study 2	Assess the effect of four 5-day treatment strategies on ADG	Daily weight gain	Pig	Mixed linear model	II
	Compare the effect of antimicrobial agent on diarrhoea prevalence the final day of the study, the excretion of <i>E. coli</i> F4 and F18, <i>L. intracellularis</i> and <i>B. pilosicoli</i> , and ADG	Daily weight gain	Pig	Mixed linear model	
		Mean qPCR results	Pen	Kruskal-Wallis rank sum test	
		Diarrhoea prevalence	Pen	Student's t-test	
	Evaluate the effect on the ADG of demonstrating faecal bacterial intestinal pathogens on the day of antimicrobial treatment initiation	Daily weight gain	Pig	Mixed linear model	
Study 3	Evaluate the pen-level agreement among three different diagnostic approaches for detecting ETEC in groups of diarrhoeic pigs	ETEC status	Pen	Fisher's exact test, Cohen's kappa coefficient	III
	Assess the value of haemolytic activity as a virulence marker	Haemolytic activity and ETEC status	Isolate	Fisher's exact test	
	Evaluate the agreement between resistance profiles of ETEC isolated from pen floor samples and from individual rectal samples	Resistance status	Pen	Visual evaluation	IV
	Compare resistance profiles from ETEC isolates and Non-ETEC isolates	Resistance status	Isolate	Generalised linear model	
		Counts of antimicrobial agent resistance per isolate	Isolate	Mixed linear model	

5 Results

This chapter summarises the main results from the three studies that make up this thesis and includes additional data not presented in the manuscripts in Chapter 6.

5.1 Results of Study 1

The results obtained in Study 1 are described in detail in Paper I.

5.1.1 Diarrhoea prevalence in nursery pigs

The main objective of this study was to estimate the diarrhoea prevalence in groups of pigs that the herd personnel assessed to be without sign of intestinal disease, and therefore not in need of antimicrobial treatment. Table 6 gives an overview of the clinical registrations of signs of intestinal diseases. A total of 2,866 pigs housed in 306 pens and from 16 herds were clinically examined, and diarrhoeic faecal droppings on the pen floor were counted. Of the included pigs, 97% were assessed to be clinically healthy by clinical inspection.

Table 6 Results of the clinical examination of 2,866 pigs

Herd	No. of pigs	% clinically healthy pigs	% pigs with hyperaemic anal region or faecal staining of hindparts	% pigs with diarrhoea	Mean diarrhoeic faecal droppings /pen
1	147	94.6	2.7	37.4	1.2
2	186	97.3	1.1	26.3	1.0
3	189	98.9	0.0	33.9	1.0
4	188	95.2	0.5	38.8	1.2
5	157	94.9	0.6	34.4	0.9
6	192	99.0	0.5	33.3	0.7
7	191	95.3	2.1	16.8	0.7
8	186	97.9	0.5	45.7	1.0
9	185	98.9	0.0	33.0	0.6
10	176	98.9	0.6	19.9	0.4
11	142	98.6	2.1	21.1	0.3
12	192	95.3	0.5	26.6	0.8
13	186	98.4	1.1	39.3	0.9
14	187	98.4	0.0	44.4	0.4
15	184	96.7	0.0	42.9	0.5
16	178	93.8	1.1	27.5	1.0
Mean	179	97.0	0.8	32.7	0.8

The proportion of pigs with clinical signs of intestinal diseases (hyperaemic anal region/faecal staining of hindparts) ranged from 0.5% to 2.7%. The within-herd prevalence of clinical diarrhoea assessed by scoring faecal samples ranged from 16.8% to 45.7%, with an average of 32.6% (CI 95% 27.9–37.3). The mean number of diarrhoeic faecal droppings per pen ranged from 0.3 to 1.2.

Figure 4 shows the relationship between the age of pigs (days post weaning) and diarrhoea prevalence.

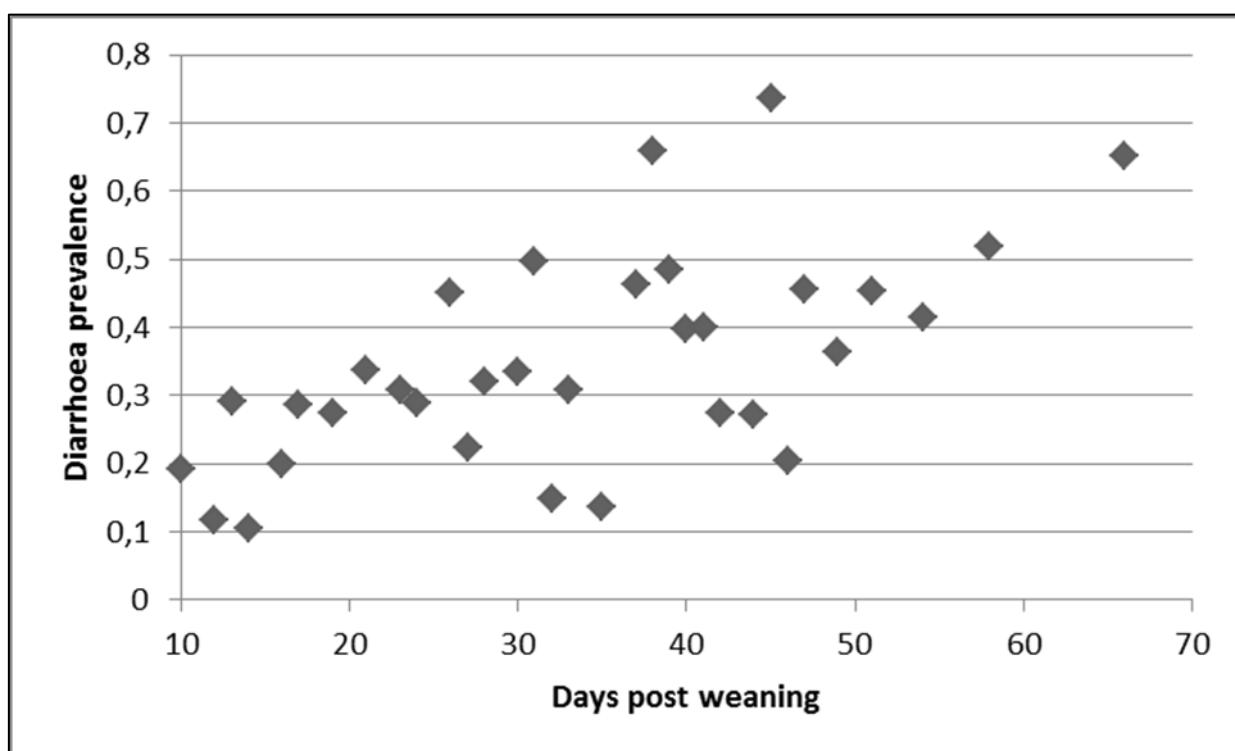


Figure 4 Plot of diarrhoea prevalence by age of pig

Diarrhoea prevalence was found to increase with the age of the pigs. There was a positive association ($p < 0.001$) between age and diarrhoea status of individual pigs, with an odds ratio of 1.04 (CI 95% 1.02–1.05) per day.

5.1.2 Prevalence of intestinal pathogens

The second objective of Study 2 was to determine the prevalence of intestinal pathogens in pigs with and without diarrhoea. A total of 256 faecal samples from 142 diarrhoeic and 114 non-diarrhoeic pigs were analysed by qPCR. *E. coli* F4, *E. coli* F18, *L. intracellularis* and *B. pilosicoli* were detected in 13.3%, 11.9%, 20.4% and 16.9% of the sampled pigs, respectively.

A correlation was observed between age of the sampled pigs and the detection of pathogens. The mean age of the pigs (days after weaning) positive for *E. coli* F4 was 27.5 days (CI 95% 24.6–30.4), *E. coli* F18 was 27.4 days (CI 95% 22.5–32.3) *L. intracellularis* was 39.3 days (CI 95% 36.1–42.5) and *B. pilosicoli* was 34.4 days (CI 95% 30.8–38.0).

No association was found between the detection of pathogens and the diarrhoea status of the individual pigs ($p>0.05$). Furthermore, in a high proportion of samples, no pathogen was detected (Table 7). At pig level, a single pathogen was detected in 34.8% of the sample, whereas multiple pathogens were detected in 12.5% of the samples. The selected pigs were housed in 127 pens distributed over 58 batches and 16 herds. At pen, batch and herd level, single pathogens were detected in 39.4% of pens, 36.2% of batches and 6.3% of herds, and multiple pathogens were detected in 12.5% of pens, 21.2% of batches and 93.7% of herds.

Table 7 Distribution of pathogen detection in random sampled non-medicated pigs, stratified by herd, batch, pen, and pig level

Pathogen detection	Pig Dia+ ^a	Pig Dia- ^a	Pig total	Pen level	Batch level	Herd level
<i>E. coli</i> F4	19(13.3%)	14(12.2%)	33(12.9%)	24(18.9%)	17(29.3%)	10(62.5%)
<i>E. coli</i> F18	17(11.9%)	12(10.5%)	29(11.3%)	22(17.3%)	17(29.3%)	11(68.8%)
<i>L. intracellularis</i>	29(20.4%)	23(20.2%)	52(20.3%)	38(29.9%)	22(37.9%)	13(81.3%)
<i>B. pilosicoli</i>	24(16.9%)	19(16.7%)	43(16.8%)	30(23.6%)	16(27.6%)	9(56.3%)
None	73(51.4%)	62(54.4%)	135(52.7%)	50(39.4%)	16(27.6%)	0(0.0%)
Single pathogen detected	51(35.9%)	38(33.3%)	89(34.8%)	50(39.4%)	21(36.2%)	1(6.3%)
1+ pathogens detected	18(12.7%)	14(12.3%)	32(12.5%)	27(21.2%)	21(36.2%)	15(93.7%)
Total	142	114	256	127	58	16

Notes. Data from qPCR analysis of faecal samples from 256 pigs. In 16 herds, 16 pigs were randomly selected from 20 pens. The 20 selected pens per herd were distributed over 2 to 6 batches per herd. ^aPrevalence of positive samples by pathogen, divided into diarrhoea status and determined by dry matter analysis

5.2 Results of Study 2

The results obtained from Study 2 are described in detail in Paper II. Three herds were included in the study and these are described in Table 8. A total of 10 batches with 1,047 pigs housed in 78 pens were included on Day 14 post weaning.

Table 8 Description of Herd A, B & C

	Herd A	Herd B	Herd C
SPF status	Blue SPF	Blue SPF	Blue SPF + Mhyo
No. of sows	700	660	660
No. of weaners	2,650	2,500	3,150
Type of feed	Home-mixed	Home-mixed	Home-mixed
Mean weight Day 14	9.39	9.84	9.16

5.2.1 Descriptive results Study 2

Daily weight gain

The main outcome of interest in Study 2 was the ADG over the period of 14 to 35 days after weaning. The overall ADG is listed in Table 9 and stratified by herd and type of treatment and strategy group.

Table 9 Results of univariable analyses of daily weight gain and explanatory variables

Explanatory variable	Daily weight gain(g)			
		n (%)	Mean	Sd
	Overall	1,047(100)	0.514	0.151
Herd				<0.001
	Herd A	392(37)	0.429a	0.143
	Herd B	447(43)	0.563b	0.136
	Herd C	208(20)	0.567b	0.124
Treatment				<0.001
	Doxycycline	449(43)	0.533a	0.145
	Tylosin	428(41)	0.504b	0.156
	No treatment	170(16)	0.487b	0.148
Strategy group				<0.001
	S1	273(26)	0.548a	0.157
	S2	270(26)	0.517b	0.159
	S3	295(28)	0.494b	0.135
	S4	209(20)	0.493b	0.144

Notes Different letters indicate a significant difference ($p < 0.05$), as per Student's t-test.

The ADG was significantly lower in Herd A than Herds B and C. No further investigation was conducted to explain this significantly lower weight gain. In the final model, the variable Herd was included as a random effect to adjust for the herd effect on weight gain. In the univariable analysis, the type of treatment was significantly associated with daily weight gain. Pigs treated with doxycycline had a significantly higher daily weight gain than pigs treated with tylosin or pigs that had not received treatment. Pigs were allocated to four different strategy groups (as previously described), and the strategy group was associated with daily weight gain. The highest

daily weight gain was observed in pigs selected for strategy group S1 compared to pigs selected for strategy groups S2, S3 and S4.

Diarrhoea prevalence

In Table 10, the mean pen-level diarrhoea prevalence assessed in indicator pigs in pens not subjected to antimicrobial treatments is displayed.

Table 10 Mean pen-level diarrhoea prevalence in pens not subjected to antimicrobial treatment

Herd	Batch	Days post weaning			
		Day 14	Day 21	Day 28	Day 35
A	1	0.07	0.26	0.25	0.25
	2	0.06	0.59	0.25	0.11
	3	0.00	0.31	0.22	0.28
	4	0.10	0.40	0.34	0.61
	Mean Herd A	0.06	0.38	0.26	0.36
B	5	0.20	0.28	0.38	0.45
	6	0.19	0.25	0.04	0.50
	7	0.23	0.07	0.34	0.25
	8	0.07	0.13	0.20	- ^a
	Mean Herd B	0.18	0.21	0.24	0.40
C	9	0.08	0.23	0.27	-
	10	0.18	0.20	0.27	0.47
	Mean Herd C	0.14	0.21	0.27	0.47
Overall mean		0.12	0.28	0.26	0.39

Notes Pen-level diarrhoea prevalence assessed in randomly selected indicator pigs. ^a All pens in batch treated with antimicrobials

The diarrhoea prevalence increased over time in all three herds. Variation was observed both between herds and between batches within the same herd.

Pathogen detection

Table 11 shows the detection of pathogens in pen floor samples analysed by qPCR in pens not subjected to antimicrobial treatment.

In total, 78 pens were included at the start of the study (Day 14). Due to scheduled pen medication according to treatment protocol, 58, 31, and 13 pens were included for analysis on Day 21, 28 and 35 after weaning, respectively.

In all three herds, *E. coli* F18 and *L. intracellularis* were the most frequently detected pathogens. Initially, *E. coli* F18 was the most frequently detected, but *L. intracellularis* was more common at the end of the study. In pens where pathogens were detected, the most common finding was single pathogen detection. When comparing pathogen detection by batch, more than one pathogen per section was most common.

Table 11 Prevalence of pathogen-positive samples detected in pen floor samples by qPCR in pens not subjected to antimicrobial treatment

Batch	Day 14 post weaning					Day 21 post weaning					Day 28 post weaning					Day 35 post weaning				
	F4 ^a	F18 ^b	Law ^c	Pilo ^d	#p ^e	F4	F18	Law	Pilo	#p	F4	F18	Law	Pilo	#p	F4	F18	Law	Pilo	#p
1	0/10 ^f	3/10	0/10	0/10	1	1/8	7/8	0/8	0/8	2	0/3	1/3	1/3	0/3	2	0/1	0/1	1/1	0/1	1
2	0/10	3/10	0/10	0/10	1	0/6	5/6	0/6	0/6	1	0/4	2/4	0/4	0/4	1	0/1	0/1	0/1	0/1	0
3	0/10	1/10	0/10	0/10	1	0/6	4/6	1/6	0/6	2	0/4	1/4	4/4	0/4	2	0/2	0/2	2/2	0/2	1
4	0/10	6/10	2/10	0/10	2	0/6	5/6	4/6	0/6	2	0/4	0/4	4/4	2/4	2	0/2	0/2	2/2	2/2	2
5	0/8	2/8	3/8	0/8	2	0/6	1/5	5/5	0/6	2	0/2	0/2	2/2	0/2	1	0/2	0/2	2/2	0/2	1
6	0/8	3/8	4/8	2/8	3	0/6	0/6	5/6	1/6	2	0/3	0/3	3/3	0/3	1	0/2	0/2	2/2	0/2	1
7	0/8	4/8	0/8	0/8	1	0/6	2/6	0/6	1/6	2	0/4	0/4	3/4	0/4	1	0/2	0/2	2/2	0/2	1
8	0/8	5/8	2/8	0/8	2	0/4	0/6	4/6	0/6	2	0/2	0/2	1/2	0/2	1	- ^g	-	-	-	-
9	0/8	5/8	0/8	0/8	1	0/4	2/6	1/6	1/6	3	0/2	0/2	2/2	0/2	1	-	-	-	-	-
10	2/8	8/8	4/8	2/8	4	0/6	0/6	3/6	3/6	2	0/3	0/3	3/3	3/3	2	0/1	0/1	1/1	0/1	1
Total	2/78	40/78	15/78	4/78		1/58	26/58	23/58	6/58		0/31	4/31	23/31	5/31		0/13	0/13	12/13	2/13	

Notes ^a *E. coli* F4, ^b *E. coli* F18, ^c *L. intracellularis*, ^d *B. pilosicoli*, ^e Number of pathogens detected per batch ^f Pens with pathogen detection/total pens sampled,

^g All pens in batch treated with antimicrobials

5.2.2 Effect of treatment strategy and type of antimicrobial on average daily weight gain

The first objective of this study was to assess the effect of four 5-day treatment strategies and two different types of antimicrobials on ADG.

A mixed linear model was constructed with daily weight gain as outcome; start weight, strategy and antimicrobial group as explanatory variables, and pen, batch and herd as random effects to account for clustering at pen, batch and herd level. The ADG was significantly correlated with strategy group ($p=0.01$), with the highest ADG observed in pens treated on Day 14 post weaning (S1) and the lowest in pens where treatment was only initiated when a clinical outbreak of diarrhoea occurred (S4). Pigs treated with tylosin had an apparent decrease in ADG of 15 g compared to pigs treated with doxycycline, but this difference was not statistically significant ($p=0.209$).

5.2.3 Effect of antimicrobial agent on diarrhoea prevalence and excretion of pathogens

The second objective of the study was to compare the effect of antimicrobial agent on the diarrhoea prevalence on the final day of the study, and the excretion of *E. coli* F4 and F18, *L. intracellularis* and *B. pilosicoli* 2 days after treatment.

The mean prevalence of diarrhoea on the final day of the study was significantly lower ($p=0.04$) in pens treated with doxycycline (0.167, 95% CI: 0.124–0.210) compared to pens treated with tylosin (0.254, 95% CI: 0.184–0.324).

E. coli F18 and *L. intracellularis* were the main pathogens detected in this study and these were used to evaluate the effect of antimicrobial agent. *L. intracellularis* excretion was significantly associated with the type of antimicrobial agent. The odds of detecting *L. intracellularis* (and specifically a high level of *L. intracellularis*) 2 days after treatment was significantly higher in pens treated with tylosin compared to pens treated with doxycycline. No effect of antimicrobial agent was observed in the excretion of *E. coli* F18. Reduced excretion of *E. coli* F18 was observed in 66 of 75 pens. The proportion of pens with a reduction in *E. coli* F18 excretion was not statistically significantly different among pens treated with tylosin (93%), doxycycline (92%; $p=0.573$) or pens receiving no antimicrobial treatment (85%; $p=0.501$).

5.2.4 Effect of demonstrating faecal bacterial intestinal pathogens at antimicrobial treatment initiation on the average daily weight gain

The third objective of the study was to evaluate the effect of demonstrating pathogens in pen floor samples at antimicrobial treatment initiation on the ADG. A total of 20 pens with 273 indicator pigs allocated for treatment on Day 14 post weaning were used in the analysis. The qPCR results of pen floor samples were dichotomised into positive and negative results. A sample was considered positive when one or more pathogens were detected in the sample. A mixed linear model was built, with ADG as the outcome, and pen, batch and herd as random effects. The qPCR results from the pen samples and antimicrobial group (doxycycline/tylosin) were the primary independent variables and were included as fixed effects. A significant difference in ADG was observed between qPCR-negative and -positive pens ($p=0.040$). Pigs housed in pens with positive qPCR results on the day of treatment initiation had an ADG of 66 g more than pigs housed in pens with a negative qPCR result.

5.3 Results of Study 3

The overall purpose of Study 3 was to investigate whether pen floor samples can be used to identify ETEC-positive diarrhoeic pigs, and in susceptibility testing for selecting antimicrobial agents for treatment. Results from Study 3 are described in detail in Papers III and IV.

Sampling was performed in a total of seven batches. Pens not subjected to antimicrobial treatment and with a diarrhoea prevalence $>25\%$ (mean=31.6%) were included, and the distribution of *E. coli* isolates by batch are listed in Table 12.

Table 12 Distribution of sampled *E. coli* isolates by batch

Batch	Sampled pens	Pig isolates	Pen isolates	Total
		Haemolytic/Non-haemolytic	Haemolytic/Non-haemolytic	
1	5	20/28	22/21	91
2	6	2/34	2/20	58
3	5	5/19	1/19	44
4	3	8/10	15/8	41
5	3	6/14	4/12	36
6	6	2/33	0/20	55
7	3	11/16	13/15	55
Total	31	54/154	57/115	380

The number of pens fulfilling the inclusion criteria varied from 3 to 6 per batch, and 10 pens were included on Day 14; 19 pens on Day 21, and 2 pens on Day 28 post weaning. *E. coli* was detected in all batches but with a large variation in the total number of isolates and type of isolate (haemolytic/non-haemolytic). All isolates were analysed for haemolytic activity and virulence genes and underwent susceptibility testing as previously described. The only adhesin gene detected in this study was F18, and STb was the most commonly detected toxin gene. A significant relationship between haemolytic activity and virulence (adhesin+toxin+) was observed with a sensitivity of 97.8% (CL95%: 92.1% - 99.7%) and a specificity of 91.8% (CL95%: 88.0% - 94.6%).

5.3.1 Agreement among three different diagnostic approaches for detecting ETEC

The first objective of the study was to evaluate the agreement between the detection of ETEC by: (D1) bacterial culture and PCR testing for virulence genes in selected *E. coli* colonies from three faecal samples per pen, which were obtained from individual pigs with clinical diarrhoea; (D2) bacterial culture and PCR testing of virulence genes in selected *E. coli* colonies from pen floor samples; (D3) qPCR testing for F18 genes in pen floor samples. Since F18 were the only adhesion fimbriae detected in this study, all isolates classified as ETEC were F18 positive. A total of five comparisons were performed and these are displayed in Table 13.

Table 13 Comparison of diagnostic approaches for detecting ETEC in pen floor and rectal faecal samples by bacterial culture and PCR or quantitative PCR

Comparison of diagnostic approaches	p-value ^e	Observed agreement (Pens with agreement/total pens)	Kappa ^f (Standard Error)
D1 ^a vs. D2 ^b	< 0.001	0.839 (26/31)	0.665 (0.179)
D1 vs. D3 ^c	0.009	0.742 (23/31)	0.488 (0.174)
D1 vs. AD3 ^d	< 0.001	0.871 (27/31)	0.728 (0.180)
D2 vs. D3	< 0.001	0.839 (26/31)	0.679 (0.176)
D2 vs. AD3	< 0.001	0.903 (28/31)	0.799 (0.179)

Notes ^a D1: Pig samples analysed by culture and real-time PCR were classified as ETEC-positive when one or more of the four potential isolates from each pig in a pen harboured both adhesin and toxin genes. ^b D2: Pen floor samples analysed by culture and real-time PCR were classified as ETEC-positive when one or more of the ten potential isolates from each pen harboured both adhesin and toxin genes. ^c D3: Pen floor samples analysed by qPCR were considered positive for fimbrial F18 genes when the amount of bacteria per gram faeces was above the detection limits (1.5×10^3 CFU per gram faeces). ^d AD3: Pen floor samples analysed by qPCR were considered positive for fimbrial F18 genes when the detection of bacteria per gram faeces was above 2×10^5 CFU per gram faeces. ^e Fisher's exact test. ^f Cohen's kappa coefficient significance value < 0.05.

Overall, a good agreement was observed in all of the five comparisons. The highest agreement between ETEC detection by culture in pig isolates (D1) and pen floor isolates was observed

when using qPCR testing of pen floor samples with a detection limit of 2×10^5 CFU per gram faeces (AD3). When comparing culture and qPCR testing of the same pen floor sample, the highest agreement was observed when using a detection limit of 2×10^5 CFU per gram faeces.

5.3.2 Antimicrobial susceptibility testing

The second objective of the study was to evaluate the agreement between resistance profiles of ETEC isolated from pen floor samples and from individual rectal samples from pigs. MIC values for 13 antimicrobial agents were obtained and clinical breakpoints were used to classify ETEC isolates as resistant or sensitive. The distribution of resistance in ETEC isolates was markedly diverse among the three study herds. In herd A, resistance was observed in eight different antimicrobial agents, compared to one and four antimicrobial agents in isolates from Herds B and Herd C, respectively. Furthermore, when comparing resistance patterns, no overlap was observed among the three study herds.

In 10 pens, ETEC was observed in both pig and pen floor samples and it was possible to compare the resistance (Table 14).

Table 14 Overview of resistance in ETEC isolates from pig and pen floor samples

Antimicrobial agent ^a	Overall resistance (%)	Agreement between pig and corresponding pen floor isolates ^b
Ampicillin	60.7	10/10
Apramycin	14.6	10/10
Gentamicin	14.6	10/10
Spectinomycin	18.0	9/10
Streptomycin	29.2	7/10
Sulphamethoxazole	69.7	10/10
Tetracycline	47.2	10/10
Trimethoprim	69.7	10/10

Notes ^a Selected antimicrobial agents with an overall resistance rate >1%. ^b **Pig resistance:** A pen was classified as resistance-positive for a specific antimicrobial class when a minimum of one ETEC isolate from one or more pigs in the pen was resistant. **Pen floor resistance:** Pen floor samples were classified as resistance-positive if a minimum of one ETEC isolate from the sample was resistant.

When comparing resistance between pig and corresponding pen floor isolates, perfect agreement was observed in all antimicrobial agents with the exception of spectinomycin and streptomycin resistance, where agreement was observed in 9 and 7 pens, respectively.

6 Papers

Paper I

Weber, N. R.; Nielsen, J. P.; Jakobsen, A. S.; Pedersen, L. L.; Hansen, C. F.; Pedersen, K. S.
Occurrence of diarrhoea and intestinal pathogens in non-medicated nursery pigs.
Acta Vet Scand (2015), 57:64

Paper II

Weber, N. R.; Pedersen, K. S.; Hansen, C. F.; Denwood, M.; Hjulsager, C. K.; Nielsen, J. P. Batch medication of intestinal infections in nursery pigs—A randomised clinical trial on the efficacy of treatment strategy, type of antibiotic and bacterial load on average daily weight gain.
Prev Vet Med 137 (2017) 69-76

Paper III

Weber, N. R.; Nielsen, J. P.; Hjulsager, C. K.; Jorsal, S. E.; Haugegaard, S.; Hansen, C. F.; Pedersen, K. S. Comparison of bacterial culture and qPCR testing of rectal and pen floor samples as diagnostic approaches to detect enterotoxigenic *Escherichia coli* in nursery pigs.
Prev Vet Med 143 (2017) 61-67

Paper IV

Weber, N. R.; Nielsen, J. P.; Jorsal, S. E.; Haugegaard, S.; Pedersen, K. S. Comparison of antimicrobial resistance in *E. coli* isolates from diarrhoeic weaned pigs and pen floor samples
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6.1 Paper I

Occurrence of diarrhoea and intestinal pathogens in non-medicated nursery pigs

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RESEARCH

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Occurrence of diarrhoea and intestinal pathogens in non-medicated nursery pigs

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Abstract

Background: Intestinal disease in nursery pigs is the most common cause of antibiotic usage in pigs in Denmark. The decision to initiate batch medication of intestinal diseases in nursery pigs is typically made by the stock personnel based on clinical assessments of pigs and counting of diarrhoeic faecal pools on the pen floor. The target population of this study was batches of nursery pigs (10–66 days after weaning) where the stock personnel assessed the pigs to be without signs of intestinal disease and therefore did not need treatment. The objective was to determine the within-herd prevalence of diarrhoea, and to determine the prevalence of *Escherichia coli* F4 and F18, *Lawsonia intracellularis* and *Brachyspira pilosicoli* by quantitative PCR in pigs with and without diarrhoea.

Results: The overall apparent prevalence of diarrhoeic pigs across sixteen herds was 32.6 % (CI 95 % 27.9–37.3). The prevalence of diarrhoea increased ($p \leq 0.001$) with age of the pigs (days after weaning) with an odds ratio of 1.04 (CI 95 % 1.02–1.05) per extra day. Diarrhoeic pools were observed in 51 % of the pens. *L. intracellularis*, *B. pilosicoli*, *E. coli* F4 and F18 were detected in 20, 17, 13 and 11 % of the 256 faecal samples analysed by quantitative PCR respectively. There was no association between detection of pathogens and diarrhoea status of the individual pigs and between detection of pathogens in a pen and diarrhoea floor pools. In 51 % of the samples from diarrhoeic pigs, pathogens were not detected. Only 5 % of the 3060 pigs examined had clinical signs of diseases other than diarrhoea.

Conclusions: One-third of non-medicated nursery pigs had diarrhoea when clinically examined even though they were assessed as healthy by stock personnel. Diarrhoeic status of the pigs and diarrhoeic pools in pen was a poor indicator of intestinal infections with *E. coli* F4 and F18, *L. intracellularis* and *B. pilosicoli* and subclinical infections were common. Therefore, clinical examination and counting of diarrhoea pools should be supported by microbiological testing as decision tools for initiation of batch treatments of intestinal infections in nursery pigs.

Keywords: *Lawsonia intracellularis*, *Brachyspira pilosicoli*, *E. coli*, Diarrhoea, Batch medication, Pigs

Background

Prudent use of antibiotics in production animals and in humans has become a scientific, political and public issue due to the risk of development of resistance in bacteria [1]. In Denmark initiatives have been implemented in order to monitor, optimise and eventually reduce antimicrobial use in pigs [2, 3]. The purpose of the “Yellow Card”—system is to minimize the use of antibiotics in pig production in Denmark, by penalising producers with a

high level of antibiotic usage. The system was introduced in the summer of 2010 and resulted in a 24.5 % reduction during 2011 [4]. This reduction in antibiotic consumption may have resulted in lower welfare and productivity because pig producers were more reluctant to treat animals that in fact required medication [5].

Intestinal disease in nursery pigs is the most common cause of antibiotic usage in pigs in Denmark and accounts for approximately 35 % of the total usage [6]. Metaphylactic batch medication is the predominant way of treatment [7]. Several bacterial pathogens have been associated with enteritis in nursery pigs. The most common pathogens associated with intestinal infections in

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nursery pigs are *Lawsonia intracellularis*, *Brachyspira pilosicoli*, *Escherichia coli* F4 and *E. coli* F18 [8–11]. A key element in prudent use of antibiotics is to use effective diagnostic decision tools for identification of batches of pigs requiring antibiotic treatment. Previous work by our group has demonstrated a 33 % mean prevalence of diarrhoea in nursery pigs at the time point when stock personnel initiated batch medication and the decision to treat is typically done by stock personnel based on assessment of diarrhoea prevalence and diarrhoeic faecal pools on the pen floor [12]. To evaluate this decision procedure, it was necessary to investigate the occurrence of diarrhoea and intestinal infections in batches of nursery pigs assessed to be healthy by the stock personnel and thereby not receiving antibiotic treatment.

The first objective of this study was to determine the within-herd prevalence of diarrhoea in nursery pigs (10–66 days after weaning) in batches of pigs where the stock personnel assessed the pigs to be without signs of intestinal disease and therefore not in need of treatment. The second objective was to determine the prevalence of *E. coli* F4 and F18, *L. intracellularis* and *B. pilosicoli* by quantitative polymerase chain reaction (qPCR) in pigs with and without diarrhoea from the same batches of nursery pigs.

Methods

All procedures involving animals were conducted in accordance with the guidelines of the Danish Ministry of Justice with respect to animal experimentation and care of animals under study.

Design and sample size

A cross sectional study of 20 Danish commercial production herds was conducted. A sample size of 200 pigs was required to determine the prevalence of diarrhoea with an allowable error of ± 0.05 with a confidence level of 95 %, given 10 % within-herd prevalence. A sample size of 128 pigs with and without diarrhoea was required to determine the prevalence of intestinal infections with an allowable error of ± 0.10 with a confidence level of 95 %, given 50 % within-herd prevalence. The sample size calculations were done using Stata IC 13 [13].

Selection of herds and pens

Producers from 20 commercial production herds previously visited in a study of diarrhoea in nursery pigs were included in the study [12]. The herds were characterised by regular therapeutic use of oral antibiotics for treatment of intestinal diseases in nursery pigs. The target population was batches of nursery pigs 10–66 days after weaning, where the stock personnel assessed the pigs to be without signs of intestinal disease and therefore

not in needed treatment the day of our visit. Herd visits were performed during a random working day. First, 20 pens were selected for clinical examination by systematic random sampling among all pens containing nursery pigs between 10 and 66 days after weaning that had not been subjected to antibiotic treatment within the last 7 days. Pens with pigs treated with antibiotics within the last 7 days were excluded together with sick and hospital pens. The number of diarrhoeic faecal pools (defined as individual loose or watery droppings) on the floor of each pen was counted, and the number of days after weaning was recorded. Ten pigs per pen were selected by systematic random sampling. The pigs were subjected to clinical examination by visual inspection and a faecal sample was obtained from each pig by collecting freshly deposited faeces or by digital rectal manipulation using a glove. Each pig could have more than one clinical registration.

Clinical scoring and dry matter content of faecal samples

Faecal samples were stored in sealed plastic containers and scored by one observer using a faecal consistency scale with four categories where score 1 and 2 represented normal faeces and score 3 and 4 diarrhoea [14]. Among the 200 faecal samples obtained from each herd, 8 diarrhoeic samples (faecal score 3 and 4) were selected by systematic random sampling. Eight non-diarrhoeic samples with faecal score 1 and 2 were randomly selected from the same pens as the diarrhoeic samples to minimise bias. Faecal dry matter (DM %) was determined in the selected samples as described by Pedersen et al. [15] and a DM % of less or equal 18 % was considered as diarrhoea.

Microbiological testing of faecal samples

Faecal samples were subjected to qPCR analysis for *B. pilosicoli*, *L. intracellularis*, *E. coli* F4 and F18 as described by Staal et al. [16]. The detection limits of the tests were 10^2 bacteria/g faeces for *L. intracellularis* and *B. pilosicoli* and 10^3 bacteria/g faeces for the 2 *E. coli* tests. A sample was considered positive when it was above the detection limits.

Statistical analysis

Chi square was used to test differences in diarrhoea prevalence between herds and age groups and between the intestinal infections of pigs with or without diarrhoea. Excretion levels of pathogens were logarithmically transformed (log 10) before analysis. A student's *t* test was used to test the difference of mean excretion of pathogens in pigs with or without diarrhoea. To test the association between age and diarrhoea status of the individual pig a generalised linear mixed model with days after weaning as explanatory variable, and herd, room,

and pen as random effects was used to calculate the odds ratios. For all statistical tests p value <0.05 was considered significant. All statistical analysis was done using R version 3.1.2 [17].

Results

Population

A total of 16 of the 20 herds were included in the study. Four herds were excluded due to changes in the pig production systems or liquidation of the production. In 3 of the 16 herds it was only possible to collect samples from 15, 16, and 15 pens, rather than the planned 20 pens resulting in 306 pens in the dataset. In each pen 10 pigs were clinically examined giving a total of 3060 pigs. From 194 of the 3060 pigs it was impossible to obtain a faecal sample and they were subsequently excluded from the analysis. The 2866 pigs included in the final dataset were housed in 62 rooms and 306 pens. A total of 256 faecal samples were analysed by qPCR with 142 samples from diarrhoeic pigs and 114 from non-diarrhoeic pigs. Samples were reclassified as diarrhoeic (DM % ≤ 18) or non-diarrhoeic following DM % analyses.

Apparent prevalence of diarrhoea

The overall apparent prevalence of clinical diarrhoea across the herds was 32.6 % (CI 95 % 27.9–37.3). The within-herd apparent prevalence of clinical diarrhoea ranged from 16.8 to 45.7 %. Diarrhoeic pigs were found in 89 % of the 306 pens examined. There was a positive association ($p < 0.001$) between days after weaning and diarrhoea status of the individual pig with an odds ratio of 1.04 (CI 95 % 1.02–1.05) per day.

Diarrhoeic pools

Diarrhoeic pools were observed in 51 % of the pens. One diarrhoeic pool was observed in 29 % of the pens and two or more pools in 22 % of the pens. The relation between diarrhoeic pools on the floor and the prevalence of pigs with diarrhoea (faecal consistency score 3 or 4) in the pen was not evident. In 49 % of the pens, diarrhoeic pools were not observed and the mean diarrhoea prevalence was 26 %. The mean diarrhoea prevalence was 37 % in pens with one diarrhoeic pool and 42 % in pens with 2 or more diarrhoeic pools.

Clinical findings

All 3060 pigs in the study were subjected to a clinical examination. A total of 183 of the pigs had clinical signs, while 2897 (95 %) of the pigs had no clinical signs (Table 1). The clinical signs most frequently found were umbilical hernia, long hair coat, inguinal hernia and contours of spinal processes. None of the mentioned clinical signs had prevalence above 1 %.

Table 1 Clinical findings other than diarrhoea in 3060 nursery pigs during clinical examination by visual inspection

Clinical sign	n
Umbilical hernia	29
Long hair coat	23
Inguinal hernia	21
Contours of spinal processes	20
Hollow lumbar region	18
Unthrifty	17
Faecal stain	13
Lameness	12
Hyperaemic anal region	10
Abdominal distension	4
Skin disease	2
Contours of the pelvis	1
Anaemic	1
Other	12
Total no clinical signs	183

Each pig may have more than one clinical registration

Microbiological findings by qPCR

The prevalence of positive samples by pathogen divided into diarrhoea status determined by DM analysis is shown in Table 2. A sample with a DM % of less or equal 18 % was considered as diarrhoea. The prevalence of the intestinal infections in different combinations is shown in Table 3. In the 256 faecal samples analysed by qPCR one or more pathogens were detected in 121 (47 %). *L. intracellularis* were detected in 52 samples (20 %), *B. pilosicoli* in 43 samples (17 %), *E. coli* F4 in 33 (13 %) and *E. coli* F18 in 29 samples (11 %). Among the positive samples 89 (74 %) contained only one pathogen whereas two or more pathogens were detected in 32 (26 %). There was no association between detection of pathogens and diarrhoea status of the individual pigs ($p > 0.05$). In 73 (51 %) of the samples from diarrhoeic pigs none of the 4 analysed pathogens were found.

In Table 4 the association between detection of one or more pathogens by qPCR and level of diarrhoeic pools from where the pigs were housed is displayed. There was no association between diarrhoeic pools and detection of pathogens ($p > 0.05$).

The mean age of pigs positive for *E. coli* F4, *E. coli* F18, *L. intracellularis* and *B. pilosicoli* was 27.5 days (CI 95 % 24.6–30.4), 27.4 days (CI 95 % 22.5–32.3), 39.3 days (CI 95 % 36.1–42.5), and 34.4 days (CI 95 % 30.8–38.0) after weaning, respectively. The excretion levels of the intestinal pathogens from positive pigs are shown in Table 5. The mean excretion level for all positive samples was 8.45 log₁₀ (CI 95 % 7.32–9.58) pathogenic bacteria/g faeces.

Table 2 Result of qPCR analysis for *Escherichia coli* F4 and F18, *Brachyspira pilosicoli* and *Lawsonia intracellularis*, in 256 faecal samples from nursery pigs

Pathogen	N (diarrhoea/ non-diarrhoea)	% of diarrhoeic pigs ^a	% of non-diarrhoeic pigs ^a	OR ^b	p value ^c
<i>E. coli</i> F4	(19/14)	13.3	12.2	1.10	0.94
<i>E. coli</i> F18	(17/12)	11.9	10.5	1.07	0.87
<i>L. intracellularis</i>	(29/23)	20.4	20.2	1.02	0.91
<i>B. pilosicoli</i>	(24/19)	16.9	16.7	1.02	0.91
1 Single pathogen detected	(51/38)	35.9	33.3	1.12	0.77
1 + pathogens detected	(18/14)	12.7	12.3	1.04	0.92
None	(73/62)	51.4	54.4	0.89	0.73
Total	(142/114)				

^a Prevalence of positive samples by pathogen divided into diarrhoea status determined by DM analysis^b Odds ratio (OR) of diarrhoea with presence of pathogens in sample^c Association tested by Chi square-test**Table 3 Simultaneous presence of the pathogens *Escherichia coli* F14 and F18, *Lawsonia intracellularis*, *Brachyspira pilosicoli* in 256 faecal samples from nursery pigs**

No. of pigs	<i>E. coli</i> F4	<i>E. coli</i> F18	<i>L. intracellularis</i>	<i>B. pilosicoli</i>
7	×	×	0	0
5	×	0	×	0
5	×	0	0	×
4	0	×	×	0
1	0	×	0	×
6	0	0	×	×
1	×	×	×	0
1	×	0	×	×
1	0	×	×	×
1	×	×	0	×

× presence of the pathogen, 0 absence of the pathogen

The mean excretion of positive samples for *E. coli* F4, *E. coli* F18, *B. pilosicoli* and *L. intracellularis* was 9.87, 7.82, 4.43, 4.67 log₁₀ bacteria/g faeces, respectively. There was no significant difference in excretion levels of the pathogens in samples from diarrhoeic pigs and non-diarrhoeic pigs [Student t test ($p > 0.05$)].

Discussion

The prevalence of clinical diarrhoea in nursery pigs in batches where the stock personnel considered the pigs to be healthy was 32.6 %. A similarly high diarrhoea prevalence of 33 % has been reported previously in batches from the same herds where the stock personnel initiated antibiotic batch medications [12]. This indicates that the actual occurrence of clinical diarrhoea might have limited influence on when the disease becomes evident for the stock person. In one study from 1998 of nursery pigs from 72 case herds, suffering from problems with diarrhoea which were treated with antibiotics, a clinical diarrhoea prevalence of 5–50 % was reported [18]. In other studies of diarrhoea in finishing pigs, the reported diarrhoea prevalence was lower. Cagienard et al. [19] reported a diarrhoea prevalence of 0.3 % in 100 kg pigs from 47 pig farms in Switzerland, whereas Stege et al. [20] reported no pigs with diarrhoea in a study of 79 finisher herds in Denmark. In another large study of Danish finishing pigs the diarrhoea prevalence as observed from outside the pen was 2.7 % [21]. A likely explanation for the different prevalence's of diarrhoea reported could be the age of the pigs and the procedure in the present study where diarrhoea status was assessed by visual inspections

Table 4 Detection of one or more pathogens by level of diarrhoeic pools from where the pigs were housed

Diarrhoeic pools per pen	No. of pens	Mean diarrhoea prevalence	Pathogen detected			Odds ratio ^a	p value ^b
			+	–	Total		
0	150	0.26	50	56	106	0.99	0.92
1	89	0.37	36	42	78	0.94	0.92
>2	67	0.42	35	37	72	1.08	0.90
Total	306	0.33	121	135	256		

^a Odds ratio (OR) of pathogen detection in pen with level of diarrhoeic pools per pen^b Association tested by Chi square-test

Table 5 Excretion levels of intestinal pathogens in positive qPCR samples from 256 nursery pigs

Intestinal pathogens	Diarrhoea	n	Min	25 % quartile	Mean	75 % quartile	Max	p value ^a
<i>E. coli</i> F4	+	19	5.74	6.66	9.59	13.89	14.55	0.64
	—	14	5.26	5.95	10.25	14.53	14.66	
<i>E. coli</i> F18	+	17	3.93	5.23	8.09	10.66	13.99	0.66
	—	12	3.74	4.31	7.44	9.66	14.02	
<i>L. intracellularis</i>	+	29	3.27	3.79	4.87	5.49	7.21	0.17
	—	23	3.27	3.70	4.43	5.06	6.57	
<i>B. pilosicoli</i>	+	24	3.27	3.79	4.59	5.37	7.37	0.15
	—	19	3.40	3.81	4.23	4.58	5.27	

^a Student's t-test of difference in mean excretion level between samples from diarrhoeic and non-diarrhoeic pigs

of the pigs, rather than faecal sampling. Only 13 pigs in the current study had had faecal contamination of the perineum indicating that use of perianal faecal staining would lead to a considerable underestimation of the diarrhoea prevalence.

The within-herd diarrhoea prevalence differed between the 16 herds. Apart from infections, factors such as levels of crude protein in the diets, weight at weaning, weaning age, and hygiene level could influence the diarrhoea prevalence [22–25]. In addition, diarrhoea prevalence was found to increase with the age of pigs which could be due to longer time at risk for developing diarrhoea, different diets and higher stocking density resulting in an increased infection pressure [26].

Decisions on antibiotic batch medication of diarrhoea are most often based on assessment of diseased pigs and by counting diarrhoeic faecal pools in the pen [12]. In this study, diarrhoeic pools on the pen floor were observed in 51 % of the pens only although diarrhoeic pigs were present in 89 % of the pens. This indicates that assessment of diarrhoea based on counting of diarrhoeic pools will likely result in a sizeable underestimation of pigs with diarrhoea.

The most frequently detected pathogen was *L. intracellularis*. The excretion level in 44 % of the pigs tested was high and above the level previously reported to be indicative of proliferative enteropathy [27–30]. For the other pathogens the excretion levels were at same level as previous reported from batches of pigs with outbreaks of diarrhoea [16].

There was no association between intestinal pathogens detected and diarrhoea status of the individual pigs, and the level of excretion was also identical between pigs with and without diarrhoea. In approximately 50 % of samples from pigs with diarrhoea, no pathogenic intestinal bacteria were detected by qPCR indicating that other causes of diarrhoea including viruses were present. Therefore, diarrhoeic status is a poor indicator of intestinal infections with *E. coli* F4 and F18, *L. intracellularis* and *B.*

pilosicoli in pigs and subclinical infections are common. Decisions on batch medication of intestinal infection in nursery pigs should be based on other indications than diarrhoea status and counting of diarrhoeic pools on the pen floor such as qPCR testing of faecal samples [7].

Our study was conducted in 2011 when the “Yellow card” system was implemented and the antibiotic consumption in pigs was reduced by approximately 25 %. This reduction could have led pig producers to be more reluctant to batch medicate animals with antibiotics that in fact required treatment. Due to the fact that the sampling of herds was not random and a small sample size of the qPCR tested faeces samples, extrapolation to the whole of the Danish pig industry should be done with caution.

Conclusions

One-third of the pigs in batches of non-medicated nursery pigs assessed 10–66 days after weaning by stock personnel to be healthy had diarrhoea and the prevalence increased with the age of the pigs. Diarrhoeic status of the pigs was a poor indicator of intestinal infections with *E. coli* F4 and F18, *L. intracellularis* and *B. pilosicoli* and subclinical infections were common. Therefore, intestinal infections were present in pigs with or without diarrhoea and the number of pigs with diarrhoea; faecal stains and diarrhoeic pools on the pen floor were inadequate as decisions tool for deciding when to treat intestinal infection using batch medication. Making decisions on batch medication of nursery pigs by assessing the number of pigs with faecal stains and counting of diarrhoeic pools on the floor will likely result in a sizeable proportion of pigs with diarrhoea not receiving treatment.

Authors' contributions

KP and JN designed the sampling protocol and selected methods; LP and AJ performed data sampling; NW performed statistical analysis; KP, JP, CFH and NW conceived the study and drafted the manuscript. All authors contributed to finalizing the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

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6.2 Paper II

Batch medication of intestinal infections in nursery pigs - A randomised clinical trial on the efficacy of treatment strategy, type of antibiotic and bacterial load on average daily weight gain

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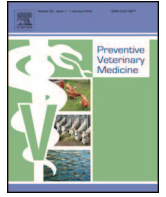
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Batch medication of intestinal infections in nursery pigs—A randomised clinical trial on the efficacy of treatment strategy, type of antibiotic and bacterial load on average daily weight gain



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ABSTRACT

Introduction: Previous research projects have demonstrated the need for better diagnostic tools to support decisions on medication strategies for infections caused by *Escherichia coli* F4 (F4) and F18 (F18), *Lawsonia intracellularis* (LI) and *Brachyspira pilosicoli* (PILO). This study was carried out as a randomised clinical trial in three Danish pig herds and included 1047 nursery pigs, distributed over 10 batches and 78 pens. The objectives of this study were: (1) to assess the effect of four 5-day treatment strategies (initiated at clinical outbreak of diarrhoea or at fixed time points 14, 21, or 28 days after weaning) on average daily weight gain (ADG); (2) to compare the effect of treatment with doxycycline or tylosine on diarrhoea prevalence, pathogenic bacterial load, and ADG; (3) to evaluate PCR testing of faecal pen floor samples as a diagnostic tool for determining the optimal time of treatment.

Results: (1) The four treatment strategies had a significant overall effect on ADG ($p = 0.01$). Pigs starting treatment 14 days after weaning had a significantly higher ADG (42 g) compared to pigs treated on day 28 ($p = 0.01$).

(2) When measured 2 days after treatment, doxycycline treatment resulted in fewer LI-positive pens ($p = 0.004$), lower excretion levels of LI ($p = 0.013$), and fewer pens with a high level of LI ($p = 0.031$) compared to pens treated with tylosine. There was no significant difference in F4, F18 and PILO levels after treatment with the two antibiotic compounds. There was a significant difference ($p = 0.04$) of mean diarrhoea prevalence on day 21 of the study between pens treated with tylosine (0.254, 95% CI: 0.184–0.324), and doxycycline (0.167, 95% CI: 0.124–0.210). The type of antibiotic compound was not found to have a significant effect on ADG ($p = 0.209$).

(3) Pigs starting treatment on day 14 in pens where F4, F18, LI or PILO were detected by qPCR on the pen floor had a statistically significant increase in ADG (66 g) compared to pigs treated on day 14 in pens where no enteric pathogens were detected ($p = 0.04$).

Conclusions: The results of this study showed that the highest ADG was achieved when treatment was initiated 14 days after weaning in pens where intestinal pathogens were detected. Doxycycline was more effective in reducing diarrhoea and LI excretion levels than treatment with tylosine.

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1. Introduction

The use of antimicrobials in livestock production is subject to continued debate due to the risk of bacterial resistance spreading to the human population (van den Bogaard and Stobberingh, 2000; Leung et al., 2011; Marshall and Levy, 2011). Denmark has a large pig industry, and antimicrobials prescribed for pigs account for 78% of the total usage for animals, corresponding to 84.8 t of

Abbreviations: qPCR, quantitative polymerase chain reaction; ADG, average daily weight gain (from 14 to 35 days after weaning); F4, *Escherichia coli* F4; F18, *Escherichia coli* F18; LI, *Lawsonia intracellularis*; PILO, *Brachyspira pilosicoli*; S1, treatment strategy 1; S2, treatment strategy 2; S3, treatment strategy 3; S4, treatment strategy 4.

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active compound (DANMAP, 2014). In 2012, 43% of active compounds prescribed for pigs were used in nursery pigs (7–30 kg live weight), of which 75% were prescribed for oral treatment of gastrointestinal disease (Jensen et al., 2014). There is great potential to reduce the total antibiotic usage by improving diagnostic criteria for batch medication of intestinal infections in nursery pigs, thereby avoiding unnecessary treatments. The most common method of treating intestinal disease in Danish nursery pigs is oral treatment for 5 days with either doxycycline or tylosine (Hybschmann et al., 2011; DANMAP, 2014; Jensen et al., 2014; EMA, 2015).

Diarrhoea has been shown to have a weak association to intestinal infection and therefore also for decisions to initiate antibiotic batch medication in pigs. Previous studies have demonstrated that bacterial enteric infections can be present within a group of pigs before the disease is clinically evident to farmers and veterinarians (Brandt et al., 2010; Paradis et al., 2012; Weber et al., 2015). It has also been demonstrated that some groups of nursery pigs experience clinical diarrhoea of non-bacterial aetiology and therefore should not be treated with antibiotics (Callesen et al., 2007; Chase-Topping et al., 2007; Thomson, 2009; Pedersen et al., 2014a). The mean bacterial load determined by qPCR testing of a pooled faecal sample for *Escherichia coli* F4 (F4) and F18 (F18), *Lawsonia intracellularis* (LI) and *Brachyspira pilosicoli* (PILO) from a group of nursery pigs can be used to determine the prevalence of bacterial enteritis/colitis (Pedersen et al., 2014b). These findings have made it possible to classify outbreaks as high or low pathogenic diarrhoea (Pedersen et al., 2014a). By classifying the pathogenicity of diarrhoeic outbreaks, it is possible to explore whether PCR testing of pooled faecal samples can be used as a decision tool for initiating batch medication.

The aim of this study was to improve the effect of antibiotic treatment for enteric infections in groups of nursery pigs. We determined the optimum time-point for initiation of batch medication, as well as the diagnostic value of using PCR testing of pooled faecal samples at the potential time of treatment. Furthermore, we compared the efficacy of batch medication with doxycycline and tylosine. The efficacy of treatment was measured as reduced diarrhoea prevalence, pathogenic bacterial load and average daily weight gain (ADG). Three different objectives were investigated in the study: **Objective 1** was to assess the effect of four 5-day treatment strategies on ADG and pathogenic bacterial load, initiated either at clinical outbreak of diarrhoea or at fixed time points 14, 21, or 28 days after weaning; **Objective 2** was to compare the effect of doxycycline and tylosine treatments on diarrhoea prevalence, pathogenic bacterial load, and ADG; **Objective 3** was to evaluate PCR testing of faecal pen floor samples at the time of treatment as a diagnostic tool for determining the optimal treatment time.

2. Methods

The study was performed as a clinical field trial approved by the Danish Medicines Agency (License no. 2013110114). Data were collected from January 2014 until October 2014.

2.1. Design

The study was a non-blinded randomised, controlled clinical trial in three herds, with a 2×4 factorial design with two antibiotics and four treatment strategies. The groups were allocated by cluster randomisation. The unit of randomisation was the pen, and the experimental unit was either the individual pig or the pen, depending on the outcome. A batch was defined as a group of nursery pigs all weaned at the same time into the same section. A total of two to four batches per herd were included 14 days after weaning and

Table 1

Distribution of pens per herd and per treatment strategy.

Strategy	Antibiotic	Herd			Pens per treatment strategy
		1	2	3	
S1	Doxycycline	4	4	2	10
S1	Tylosine	4	4	2	10
S2	Doxycycline	4	4	2	10
S2	Tylosine	4	4	2	10
S3	Doxycycline	5	4	2	11
S3	Tylosine	5	4	2	11
S4	Doxycycline	4	3	1	8
S4	Tylosine	4	3	1	8
Pens per herd		34	30	14	

followed for 21 days. Batches with mixed age groups or treatments of unexpected diseases were excluded.

2.2. Sampling procedures

A total of 78 pens were included in the study (Table 1). Within a batch, four double pens sharing the same feeder were randomly selected. A total of 15 pigs from each of the selected double pens were selected by systematic random sampling. If there were fewer than 15 pigs in the selected pen, all pigs were selected. All trial pigs were ear-tagged with a unique ID number. Pooled faecal pen floor samples were collected from each study pen at day 14, 21, 28 and 35 post weaning. Excretion level of F4, F18, LI and PILO analysed by qPCR in the pooled faecal samples was used to evaluate pathogenic bacterial load.

To address Objective 1, randomly selected double pens were allocated to four different treatment strategies. The four strategies tested were: **strategy 1 (S1)**: 5 days of antibiotic treatment initiated 14 days after weaning; **strategy 2 (S2)**: 5 days of antibiotic treatment initiated 21 days after weaning, or at an earlier time point if there was an outbreak of clinical diarrhoea; **strategy 3 (S3)**: 5 days of antibiotic treatment initiated 28 days after weaning, or at an earlier time point if there was an outbreak of clinical diarrhoea; **strategy 4 (S4)**: 5 days of antibiotic treatment only initiated in response to an outbreak of clinical diarrhoea. An outbreak of clinical diarrhoea was defined by the fulfilment of one of the following criteria: $\geq 50\%$ of pigs with diarrhoea; $>50\%$ of pigs treated individually for intestinal disease. Regardless of predetermined time point for treatment all pens were treated for animal welfare reasons when a diarrhoeic outbreak occurred.

To address Objective 2, two different active compounds (doxycycline/tylosine) were used in parallel throughout the study. Pens were assigned at random to antibiotic type when included at the start of the study. To address Objective 3, the qPCR test results from samples collected at the day of treatment 14 days after weaning (S1) were used to classify the study pens according to the load of pathogenic bacteria in the pooled faecal pen floor sample collected on the day that treatment was initiated. This classification was used in the subsequent statistical analysis to assess the effect of faecal bacterial intestinal pathogens at the day of initiation of treatment on ADG in the following 21 days.

2.3. Sample size considerations

Sample size calculations were performed using formulae for differences in mean between two groups. The groups were allocated by cluster randomisation (at pen level), but weight gain was measured in the individual pig. The study was designed to detect a 50 g ADG difference between pigs subjected to different treatment strategies. When taking into account the effect of clustering (as described by Dohoo et al., 2009), each treatment strategy required 180 pigs (Dohoo et al., 2009).

2.4. Herds

Potential study herds were selected from herds serviced by two veterinary practices in the eastern part of Denmark. Herds free of porcine reproductive and respiratory syndrome virus, Edema disease, *Brachyspira hyodysenteriae*, salmonellosis, atrophic rhinitis, and other acute diseases with a risk of medication were included. Vaccination against LI was an exclusion criterion. High pathogenic diarrhoea was an inclusion criterion and was defined as an outbreak with ≥ 1.5 diarrhoeic pools per pen and faecal pool samples containing $\geq 35,000$ bacteria per g of faeces, calculated as the sum of F4, F18, PILO and LI per g faeces (Pedersen et al., 2014a). Three herds were included in the study. All herds had all-in all-out batch production in sectioned compartments with 2300 to 3600 pen places per herd. The flooring consisted of one third solid floor and two thirds slatted floor. Pigs per pen ranged for 10–40 pigs. Pig density and layout of pens was similar in all three herds. The feed fulfilled the Danish nutrient standards (SEGES-VSP, 2015) and was home-mixed, formulated with wheat, barley and soybean-meal as the main ingredients. The nursery pigs were crossbred Yorkshire/Landrace and Duroc. All three herds were participating in the Danish Specific Pathogen Free system (SPF); (SPF-sus, 2015) and were all declared free of *Actinobacillus pleuropneumoniae* type 2, 6 and 12, porcine reproductive and respiratory syndrome virus, mange mites and lice. Herds 1 and 2 were declared free of *Mycoplasma hyopneumoniae*, unlike Herd 3, which was positive and using a vaccination programme to control the infection. To control post-weaning colibacillosis, all herds used 3000 ppm zinc oxide in the feed for the first 14 days after weaning. During the field trial, each herd was visited for clinical examination once a week. All pigs were weighed at the start of the trial (14 days after weaning) and at the end of the trial (35 days after weaning), using a scale (“Bjerringbrovægt 1298GE”) with a precision of 100 g.

2.5. Assessment of faecal consistency

Faecal samples were collected from each pig by digital rectal manipulation using a gloved hand at the start of the study, on the day the pigs were treated and at the end of the study. The faecal samples were scored by one observer using a faecal consistency scale with four categories, where scores 1 and 2 represented normal faeces and score 3 and 4 represented diarrhoea (Pedersen and Toft, 2011).

2.6. Laboratory analyses

Pooled faecal pen floor samples were collected by swiping a gloved hand over the slatted floor once a week from every study pen, and the collected faeces were stored in sealed plastic containers. Cooled samples were transported (in a polystyrene box with freezing elements) to the Danish National Veterinary Institute for further laboratory analyses. Approximately 1 g of the pooled faecal samples was homogenised in a stomacher for 1 min with phosphate buffered saline (PBS) to obtain a 10% (w/v) faecal suspension. An aliquot of the suspension was transferred to a 2 ml microfuge tube and stored in a freezer at minus 20 °C until DNA extraction, as previously described by (Pedersen et al., 2012). DNA was stored in a minus 20 °C freezer until the F4, F18, PILO and LI content was quantified by qPCR, as previously described by (Stahl et al., 2011) with the exception that standard curves for quantification were prepared from DNA extracted from faeces spiked with 10-fold dilution series of the corresponding pathogen, using the same extraction procedure as for the faecal specimens (Pedersen et al., 2012). Detection limits per gram faeces were 5.7×10^4 colony-forming units (CFU) for F4, 1.5×10^3 CFU for F18, 2×10^3 bacteria for LI and PILO. Linear ranges were 5.7×10^9 – 5.7×10^5 CFU/g faeces

for F4, 1.5×10^{10} – 1.5×10^5 CFU/g faeces for F18, 2×10^8 – 2×10^4 bacteria/g faeces for LI and 2×10^8 – 2×10^4 bacteria/g faeces for PILO.

2.7. Treatments

Doxycycline hydrate was used in the trial at a dosage of 12.5 mg per kg bodyweight, as recommended by the supplier (Soludox Vet.[®], Dechra Veterinary Products A/S). Tylosine tartrate was used in the trial at the recommended dosage of 7.5 mg per kg bodyweight (Tylan[®] Vet., ELANCO Animal Health). Both antibiotics were administered via a water trough at pen level. The daily dose was divided equally over two daily administrations in order to increase the chances of uniform dosing. Due to legal regulation, pigs had access to fresh water via drink nipples during the treatment period. Oxytetracycline (Engemycin[®] Vet. 100 mg/ml, MSD Animal Health), at a standard dose of 10 mg per kg bodyweight was used for 3 days for individual treatment of intestinal disease in pens allocated to pen treatment by doxycycline. Oxytetracycline was chosen because no doxycycline products were registered for injection in Denmark. In study pens allocated to pen treatment by tylosine, any individual cases of intestinal disease were treated using tylosine (Tylan[®] Vet. 200 mg/ml, ELANCO Animal Health) with a standard dose of 10 mg per kg bodyweight for 3 days. Throughout the study period, all pen treatments were performed according to the study protocol. Pen treatments were initiated after the predetermined day according to protocol, or at an outbreak of clinical diarrhoea defined by the fulfilment of one of the following criteria: $\geq 50\%$ of pigs with diarrhoea or $>50\%$ of pigs individually treated for intestinal disease. The farmer/stockman was allowed to treat individual pigs with clear clinical signs of intestinal or other diseases. The criteria for individual treatment of intestinal disease were: observed defecation of watery faeces; a line of watery faeces in the anal region; marked loss of body condition. If the disease progressed, the pigs were weighed and removed from the study.

2.8. Statistical analyses

Statistical analyses were performed in R version 3.1.2 (R-Core-Team, 2014), with mixed models implemented using the lme4 and lmerTest packages (Bates et al., 2015; Kuznetsova et al., 2015). The effect of the four treatment strategies for 5-day treatments with doxycycline or tylosine (Objectives 1 and 2) was assessed using a mixed linear model (model 1) with ADG as the outcome, and pen, batch and herd as random effects to account for clustering at pen, batch and herd level. ADG was calculated by subtracting the start weight (bodyweight on day 14) from the end weight (bodyweight on day 35). Least squares means (lsmeans) included in the lmerTest package were used to summarise the effect of the explanatory variables on the outcome in the mixed model. Start weight, gender, faecal status, bacterial load, treatment strategy (S1–S4) and antibiotic group (doxycycline/tylosine) were individually screened as potential explanatory variables by univariable linear regression. Candidate variables with a p -value < 0.1 were used in the multivariable linear model to investigate the association with ADG. Previous eliminated variables were reintroduced to the model to control for confounding. A variable was considered to be a confounder if the estimates of the significant variables changed by 25%. Eliminated variables that were identified as confounders were retained in the final model. The measurement of difference in pathogenic load of pathogenic bacteria between strategy group and antibiotic group was tested by Kruskal-Wallis rank sum test.

The association between demonstration of faecal bacterial intestinal pathogens and ADG (Objective 3) was analysed using data from pens selected for S1 to build a mixed linear model (model

Table 2

Detection and excretion levels of pathogens in faecal pen floor samples by qPCR, and diarrhoea prevalence in non-treated pens.

Days after weaning	14			21			28			35		
Pathogen detected												
<i>E. coli</i> F4	2/78 ^a	2.6% ^b		1/58	1.7%		0/31	0%		0/13	0%	
<i>E. coli</i> F18	40/78	51.3%		26/58	48.3%		4/31	12.9%		0/13	0%	
<i>L. intracellularis</i>	15/78	19.2%		23/58	39.7%		23/31	74.2%		12/13	92.3%	
<i>B. pilosicoli</i>	4/78	5.1%		6/58	10.3%		5/31	16.1%		2/13	15.4%	
No pathogen detected	32/78	41.0%		11/58	19.0%		5/31	16.1%		1/13	7.7%	
Excretion level of positive samples (log ₁₀ bacteria/g faeces)	Quartiles			Quartiles			Quartiles			Quartiles		
	25%	50%	75%	25%	50%	75%	25%	50%	75%	25%	50%	75%
<i>E. coli</i> F4	5.55	5.66	5.82	–	5.98	–	–	–	–	–	–	–
<i>E. coli</i> F18	5.62	5.96	6.38	5.40	6.41	7.27	6.69	7.13	8.00	–	–	–
<i>L. intracellularis</i>	3.16	4.01	5.07	3.61	4.76	5.51	4.79	5.85	7.01	6.09	6.70	7.03
<i>B. pilosicoli</i>	2.98	3.38	3.96	3.19	3.61	3.89	3.09	4.46	5.12	4.33	4.72	5.12
Pen-level diarrhoea%	0.00	0.09	0.18	0.13	0.27	0.40	0.20	0.27	0.35	0.25	0.45	0.50

Results of qPCR analysis and diarrhoea prevalence from pens examined weekly at day 14, 21, 28, and 35 post weaning. All pen floor samples collected before an antibiotic treatment was labelled as samples from non-treated pens. Pen floor samples collected after treatment are not included.

^a Pens with pathogen detection.

^b Prevalence of positive pens.

2) with ADG as the outcome, and pen, batch and herd as random effects. The qPCR results from the pen samples and antibiotic group (doxycycline/tylosine) were the primary independent variables and were included as fixed effects. Assumptions for linear regression were assessed visually using normal quartile plots of residuals.

3. Results

3.1. Descriptive results

Two batches from herd 3 were excluded due to a fire in the herd facilities, and four pens were excluded due to a failure to record diarrhoeic outbreaks. Data from 12 pigs were missing due to death or movement to other sections. A total of ten batches (four batches from herd 1 and 2 and two batches from herd 3) with 78 pens containing 1047 pigs from the three study farms were included in the statistical analyses. The average number of pigs per pen was 23.7 (min = 10, max = 40). Twenty pens were selected for S1 and all were treated 14 days after weaning. Twenty pens were selected for S2, and 18 of these pens were treated 21 days after weaning, while treatment in two pens was initiated an earlier time point due to a clinical outbreak of diarrhoea. A total of 22 pens were selected for S3 and 17 of these were treated 28 days after weaning, while treatment was initiated at an earlier time point due to a clinical outbreak of diarrhoea in five pens. Sixteen pens were selected for S4. No treatment was initiated in 13 of these, while in the remaining three pens, treatment was initiated due to a clinical outbreak of diarrhoea.

3.1.1. Excretion of intestinal pathogenic bacteria and diarrhoea prevalence

Diarrhoea prevalence, detection and excretion level of F4, F18, LI and PILO in pen floor faeces sampled once a week from non-treated pens are shown in Table 2. In all three herds, F18 and LI were the most frequently detected pathogens. Initially, F18 was the predominant pathogen, but LI was more common at the end of the study period. F18 was most frequently detected on day 14 (51.3%) and day 21 (48.3%), and LI were detected on day 14, 21, 28 in 19.2%, 39.7%, 74.2% and 92.3% pens, respectively. PILO was detected at low frequency over time, and F4 was rarely found. No pathogen was detected on day 14, 21, 28 and 35 in 41.0%, 19.0%, 16.1% and 7.7% of pens, respectively. The diarrhoea prevalence at pen level increased over time from a median diarrhoea prevalence of 0.09 on

day 14–0.45 on day 35. The excretion level from positive samples showed an increase over time for all four pathogens.

Table 3 shows the total bacterial load of the most frequently detected pathogens (F18 and LI) stratified by strategy and antibiotic groups. The total bacterial load was calculated at pen level by the sum of four faecal pen floor samples collected weekly on day 14, 21, 28, and 35 post weaning. The total excretion level of F18 was significantly higher in S4 compared to the other strategy groups. There was no significant difference of median bacterial load of F18 between the two antibiotic groups ($p = 0.703$). There was no significant difference in the median total bacterial load of LI between the four strategy groups ($p = 0.335$), whereas there was a significant difference ($p = 0.03$) in the median total bacterial load of LI between the two antibiotic groups (doxycycline = $10^{4.79}$ LI bacteria/g faeces, tylosine = $10^{6.08}$ LI bacteria/g faeces).

3.2. Analytical results

3.2.1. Effect of treatment strategy and type of antibiotic on average daily weight gain

The estimates from the final model for ADG (Model 1) and least squares means of ADG in strategy and antibiotic groups are presented in Table 4. The variables included start weight, strategy and antibiotic group. Antibiotic group was included despite failing to meet the univariate criteria for inclusion ($p < 0.1$) because it was our primary variable of interest. Average daily weight gain was significantly correlated with strategy group ($p = 0.01$), with the highest ADG observed in S1 and the lowest in S4. There was a significant difference in ADG between S1 and S3 and S4, as tested by least squares means. The ADG of pigs selected for S3 and S4 were 42 g and 56 g lower, respectively, than pigs selected for S1. Pigs treated with tylosine had an apparent decrease in ADG of 15 g compared to pigs treated with doxycycline, but this difference was not statistically significant ($p = 0.209$). The intraclass correlation coefficient (ICC) values showed a 12.3% variation between the three herds, which meant that most of the variation (87.7%) was within herds. To control for the effect of pens treated due to clinical outbreak at an earlier time point then predetermined by the strategy groups on ADG, the model was run again where these pens were excluded. The estimates of the reduced model did not change markedly that could interfere on the conclusions of the effect of the main variables, strategy and antibiotic group on ADG.

Table 3Total bacterial load of *L. intracellularis* and *E. coli* F18.

Treatment strategy group	<i>L. intracellularis</i>				<i>E. coli</i> F18			
	Positive/total pens	Quartiles			Positive/total pens	Quartiles		
		25%	50%	75%		25%	50%	75%
S1	16/20	4.65	5.68 ^{a,**}	7.48	18/20	5.71	6.26 ^a	6.74
S2	13/20	5.17	6.08 ^a	7.71	18/20	5.49	5.92 ^a	6.72
S3	20/22	4.48	6.02 ^a	7.52	15/22	5.61	5.97 ^a	6.87
S4	14/16	5.82	6.95 ^a	7.55	12/16	6.77	6.98 ^b	7.55
Antibiotic group								
Doxycycline	22/33	3.73	4.79 ^a	6.16	28/33	5.67	6.39 ^a	6.88
Tylosine	29/32	4.99	6.08 ^b	6.83	26/32	5.61	5.92 ^a	6.75

^a = Total bacterial load calculated by the sum of four faecal pen floor samples collected weekly at day 14, 21, 28, and 35 post-weaning from positive pens (log10 bacteria/g faeces).

^{**} = Different letter indicates significant difference ($p < 0.05$) tested by Kruskal-Wallis rank sum test.

Table 4

Estimates for fixed effects, intraclass correlation coefficients (ICC) for random effects and estimated means from a linear mixed model for average daily weight gain from 14 to 35 days after weaning (kg).

Fixed effects	Estimate (β_x)	Std. error	95% CI	p-value	Least squares means	SEM
Intercept	0.131	0.042	0.026; 0.237	0.029		
Start weight	0.045	0.002	0.026; 0.237	<0.000		
Strategy group				0.010		
S1	–				0.552 ^{a,*}	0.037
S2	–0.028	0.016	–0.060; 0.011	0.086	0.524 ^{ab}	0.037
S3	–0.042	0.016	–0.074; –0.011	0.0106	0.510 ^b	0.038
S4	–0.056	0.017	–0.091; –0.022	0.002	0.496 ^b	0.038
Antibiotic group				0.209		
Doxycycline	–				0.528 ^a	0.036
Tylosine	–0.015	0.012	–0.038; 0.008		0.513 ^a	0.036
Random effects	Variance	Std. dev.	ICC(%)			
Herd	0.002	0.044	12.3			
Batch	0.001	0.026	4.3			
Pen	0.004	0.059	22.6			
Residuals	0.009	0.100	60.5			

Model 1. “–” Indicates reference.

^{*} = Different letter indicates significant difference ($p < 0.05$) tested by lsmeans.

3.2.2. Faecal excretion of *Lawsonia intracellularis* and diarrhoea prevalence after treatment with doxycycline and tylosine

Faecal excretion of LI analysed by qPCR from pooled pen floor samples is shown in Table 5. On the day of treatment initiation, LI was detected in 12 of the 33 pens treated with doxycycline (median excretion = $10^{5.8}$ LI bacteria/g faeces) and in 14 of the 32 pens treated with tylosine (median excretion = $10^{5.7}$ LI bacteria/g faeces). At the second sampling (i.e. 2 days after treatment), LI was detected in 7 of the 33 pens treated with doxycycline (median excretion = $10^{3.5}$ LI bacteria/g faeces) and in 18 of the 32 pens treated with tylosine (mean excretion = $10^{5.8}$ LI bacteria/g faeces). There was a significant association ($p = 0.003$) of detection of LI ($> 2 \times 10^3$ bacteria/g faeces) 2 days after treatment between the two antibiotic groups with an odds ratio of 4.78 (95% CI: 1.67–14.96) in pens treated with tylosine. There was also an association ($p = 0.03$) of detection of high LI levels ($> 10^6$ bacteria/g faeces) 2 days after treatment between the two treatment regimens with an odds ratio of 10.67 (95% CI: 1.78–204.83) in pens treated with tylosine. There was also a significant difference ($p = 0.04$) between pens treated with tylosine (0.254, 95% CI: 0.184–0.324), and doxycycline (0.167 95% CI: 0.124–0.210) in the mean prevalence of diarrhoea on the final day of the study.

3.2.3. Association between bacterial intestinal pathogen load at initiation of treatment and ADG

Table 6 shows the estimates for the mixed linear model with mean ADG as outcome (Model 2). Data for this model are a subset of the whole dataset of pigs treated on day 14 after weaning (S1). In the final mixed linear model, the qPCR results for detection of bacterial

intestinal pathogens were dichotomised into positive or negative results to ensure a sufficient number of pigs were included in each group for the analysis. A qPCR sample was classified as positive if the sample was positive for one or more of the four analysed pathogens. After adjusting for herd, batch, pen, start weight and type of antibiotic treatment, there was a significant difference in ADG between qPCR negative and positive pens ($p = 0.040$). Pigs treated in pens with a positive qPCR pen sample had an ADG increase of 66 g compared to pigs treated in pens with a negative qPCR sample.

4. Discussion

The main finding of this study was that the time of treatment affected the ADG. In general, the earlier pigs were treated (starting 14 days after weaning), the higher the ADG; pigs treated 14 days after weaning (S1) had significantly higher ADG than pigs treated on day 21 or day 28. All pigs in S1 were treated 14 days after weaning, and no other clinical parameters were taken into account when deciding upon the initiation of the pen treatment. The pigs treated on day 14 were characterised by having the lowest diarrhoea prevalence at pen level and a lower level of intestinal pathogenic bacteria at the day of treatment initiation compared to pigs treated on day 21 or 28. This study also demonstrated the effect of detecting intestinal pathogens on the day of treatment initiation on the ADG of the pigs treated. Pigs housed in pens where no pathogens were detected on the day of treatment initiation had a significantly lower ADG than those housed in pens where one or more pathogens were detected. Overall, these findings show that antimicrobial treatment had the greatest effect on ADG in the pens where pigs excreting intesti-

Table 5
Faecal excretion of *Lawsonia intracellularis* analysed by qPCR from pooled pen floor samples.

	Doxycycline	Tylosine	p-value	Odds ratio
<i>L. intracellularis</i> detection at initiating treatment				
Positive pens	12/33	14/32	0.723 ^a	
Median excretion of positive pens (bacteria/g faeces)	10 ^{5.8}	10 ^{5.7}	0.837 ^b	
<i>L. intracellularis</i> detection 2 days after last treatment (<2 × 10 ³ bacteria/g faeces)				
Positive pens	7/33	18/32	0.004 ^a	4.78
Median excretion of positive pens (bacteria/g faeces)	10 ^{3.5}	10 ^{5.8}	0.013 ^b	
Detection of high level of <i>L. intracellularis</i> (>10 ⁶ bacteria/g faeces) excretion 2 days after treatment				
Positive pens	1/33	8/32	0.031 ^a	10.67
Reduction of <i>L. intracellularis</i> excretion from initiation of treatment and 2 days after treatment ^c				
Pen with reduction	11/12	7/14	0.06 ^a	0.09

Pens were randomly selected for treatment with 5 days of doxycycline or 5 days of tylosine.

a = Chi²-test, b = Kruskal-Wallis rank sum test, c = faecal excretion of *Lawsonia intracellularis* was measured on the day of the first treatment and again 2 days after the last treatment.

Table 6
Estimates for fixed effects, intraclass correlation coefficients (ICC) for random effects and estimated means from a linear mixed model for average daily weight gain from 14 to 35 days after weaning (kg) in pigs selected for Treatment strategy 1 (S1).

Fixed effects	Estimate (βx)	Std. error	95% CI	p-value	Least squares means	SEM
Intercept	0.009	0.056	−0.103; 0.136	0.873		
Start weight	0.053	0.004	0.044; 0.061	<0.000		
qPCR pen sample				0.040		
Negative	–				0.503	0.036
Positive	0.066	0.030	0.002; 0.136		0.569	0.031
Treatment group				0.557		
Doxycycline	–				0.543	0.032
Tylosine	−0.013	0.021	−0.058; 0.033		0.520	0.032
Random effects	Variance	Std. dev.	ICC(%)			
Herd	0.002	0.043	12.7			
Batch	0.001	0.038	11.5			
Pen	0.002	0.040	15.0			
Residual	0.007	0.086	60.8			

Model 2: Data from a subset of the whole dataset of pigs selected for Treatment strategy 1 (S1).

“–” Indicates reference.

nal pathogen were treated early, when clinical diarrhoea was still at a low level. This is in accordance with previous findings that have shown subclinical enteric infections to be common, so using clinical diarrhoea in the decision to initiate treatment can be problematic (Jacobson et al., 2003; Paradis et al., 2012; Weber et al., 2015). Interestingly, when treatment was initiated in pens without pathogenic bacteria, the pigs performed poorer than pigs in the pens where pathogenic bacteria were detected. The most plausible explanation for this finding might be that the antibiotics were used at a time point where they did not affect intestinal pathogens. Only 5 days of antibiotic treatment was used throughout the study period. Therefore, pens treated at day 14 and without detection of pathogenic bacteria could have experienced intestinal infections after the antibiotic treatment that might have resulted in a lower ADG. The ADG of pigs treated at day 14 without any detectable pathogens were at a similar level as pigs treated at day 28 or later which may support the idea that these pens were treated before an infection occurred in the pigs. This demonstrates the diagnostic value of testing faecal pen floor samples at the time of treatment as a tool for initiating antibiotic treatment. Due to the small sample size, it was not possible to investigate the effect of different excretion levels of pathogenic bacteria on ADG, thereby determining a critical threshold of pathogenic bacteria excretion. If qPCR analyses can be performed in real-time at herd facilities in the future, it may be possible to determine a critical threshold for the level of pathogenic bacteria excretion for the initiation of treatment.

In this study, there was no significant difference in ADG between pigs treated with doxycycline and tylosine, but doxycycline had a better effect on LI excretion after treatment, total bacterial load

and diarrhoea prevalence. A previous study showed that treatment with 8 mg tylosine tartrate per day for 7 days via drinking water could reduce the clinical signs and lesions and improve the rate of growth in nursery pigs challenged with LI (Paradis et al., 2004). In a field study from 2000 in Greece, in-feed treatment of 250 ppm doxycycline for 14 days significantly reduced the prevalence of LI and diarrhoea and improved the rate of growth, thus supporting the results of the current study (Kyriakis et al., 2002). Although this study demonstrated no difference in ADG between doxycycline or tylosine treatments, the results showed that high-level LI remains in faeces after treatment with tylosine. Tylosine tartrate and chlortetracycline have shown high intracellular and extracellular activity against LI (Wattanaphansak et al., 2009; Yeh et al., 2011). The high level of LI found in this study 2 days after treatment with tylosine was therefore surprising. The authors are not aware of any research into the antimicrobial susceptibility of doxycycline against LI to support the findings of doxycycline effectiveness in reducing LI excretion presented in this study. However, the effect of oxytetracycline treatment has recently been demonstrated. In a randomised clinical trial, 5 days of water medication with a dose of 5 mg to 20 mg oxytetracycline per kg bodyweight resulted in reduced diarrhoea and LI excretion after treatment (Larsen et al., 2016).

The application of medication via a water trough could influence the results since the pigs also had access to fresh water during the medication period, and might have preferred the fresh water to the medicated water. However, both antibiotic compounds were administered in the same way, making a comparison reasonable. A limitation of the study is the short study period of 14–35 days

after weaning. All pigs received only 5 days of antibiotic treatment throughout this period and therefore the lowest amount of antibiotics were used in pigs with a lower bodyweight that were treated early. Due to the risk of antibiotic treatments against enteric diseases recurring after the study period, it was not possible to evaluate the effect of the four treatment strategies on the rate of growth and total antibiotic usage for the whole nursery period of approximately 8 weeks after weaning. The aim of this study was to evaluate the effects of antibiotic treatment on reducing intestinal infections and thereby improving productivity. Other factors that can reduce the infection pressure, such as improvement of management and biosecurity, were not investigated.

Difference in concentration of the two types of drugs was the main reason why the study was not blinded. The parameter “weight before treatment” was used to calculate the correct dose of either doxycycline or tylosine and thereby made blinding difficult. To eliminate investigator bias we used objective parameters for our outcome variables; body weight and faecal dry matter measured using a scale, and bacterial intestinal pathogens demonstrated by qPCR.

This study demonstrated that diagnosing intestinal infections in groups of pigs before clinical signs are evident using pooled pen floor samples tested by qPCR can be used to support the decision for initiation of antibiotic treatment. Diagnosing intestinal infections by this method gives the advantage of achieving a better productivity and avoiding unnecessary treatments, thereby reducing the antibiotic usage to minimize the development of antibiotic resistance.

Three commercial pig herds were used in this study which was representative for typical Danish productions of nursery pigs in accordance to factors which could influence the conclusion of the study; health status, enteric pathogen profile, usage of in-feed zinc oxide, feeding strategy, and antibiotic usage. Variation within herds and within the EU swine population of the listed factors should be taken into consideration when interpreting the conclusions of this study.

5. Conclusion

The strategy resulting in the highest ADG was treatment 14 days after weaning in pens where *Escherichia coli* F4, F18, *Lawsonia intracellularis* or *Brachyspira pilosicoli* were detected by qPCR. Median diarrhoea pen-level prevalence at this time point was 0.09. There was no significant difference in ADG between treatment with doxycycline or tylosine, yet doxycycline was more effective in reducing LI excretion and diarrhoea prevalence after treatment.

Competing interests

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of this paper.

Authors' contributions

JPN, CFH, KP and NW designed the sampling protocol and selected methods; CH conducted the qPCR analyses; NW performed the data sampling; MD and NW performed the statistical analysis; JPN, CFH, KP, MD, CH and NW devised the study and drafted the manuscript. All authors contributed to finalising the manuscript. All authors read and approved the final manuscript.

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6.3 Paper III

Comparison of bacterial culture and qPCR testing of rectal and pen floor samples as diagnostic approaches to detect enterotoxigenic *Escherichia coli* in nursery pigs

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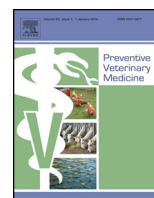
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Comparison of bacterial culture and qPCR testing of rectal and pen floor samples as diagnostic approaches to detect enterotoxigenic *Escherichia coli* in nursery pigs

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ABSTRACT

Enterotoxigenic *E. coli* (ETEC) are a major cause of diarrhoea in weaned pigs. The objective of this study was to evaluate the agreement at pen level among three different diagnostic approaches for the detection of ETEC in groups of nursery pigs with diarrhoea. The diagnostic approaches used were: bacterial culturing of faecal samples from three pigs (per pen) with clinical diarrhoea and subsequent testing for virulence genes in *E. coli* isolates; bacterial culturing of pen floor samples and subsequent testing for virulence genes in *E. coli* isolates; qPCR testing of pen floor samples in order to determine the quantity of F18 and F4 genes. The study was carried out in three Danish pig herds and included 31 pens with a pen-level diarrhoea prevalence of > 25%, as well as samples from 93 diarrhoeic nursery pigs from these pens. All *E. coli* isolates were analysed by PCR and classified as ETEC when genes for one or more adhesin factors and one or more enterotoxins were detected. Results: A total of 208 *E. coli* colonies from pig samples and 172 *E. coli* colonies from pen floor samples were isolated. Haemolytic activity was detected on blood agar plates in 111 (29.2%) of the 380 colonies that were isolated. The only adhesin factor detected in this study was F18. When comparing bacterial culture or qPCR testing of pen floor samples with detection of ETEC-positive diarrhoeic pigs by culture, agreement was found in 26 (83.9%, Kappa = 0.665) and 23 (74.2%, Kappa = 0.488) of the pens, respectively. Agreement was observed between the detection of ETEC by bacterial culture and qPCR in the same pen floor sample in 26 (83.9%, Kappa = 0.679) pens. Conclusion: We observed an acceptable agreement for the detection of ETEC-positive diarrhoeic nursery pigs in pen samples for both bacterial culture of pen floor samples and qPCR. This study showed that both bacterial culture and qPCR testing of pen floor samples can be used as a diagnostic approach for detecting groups of ETEC-positive diarrhoeic nursery pigs.

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Abbreviations: ETEC, enterotoxigenic *Escherichia coli*; PWD, post-weaning diarrhoea; qPCR, quantitative polymerase chain reaction; F4, *Escherichia coli* F4; F18, *Escherichia coli* F18; LI, *Lawsonia intracellularis*; PILO, *Brachyspira pilosicoli*.

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1. Introduction

Enteric diseases involving diarrhoea in nursery pigs are a major cause of antibiotic treatments in commercial pig production worldwide and have been subject to considerable research during recent years (Nagy and Fekete, 1999; Heo et al., 2013). Enteric diseases are endemic in many farms and can lead to major economic losses (Vondruskova et al., 2010; Fairbrother and Gyles, 2012). Bacterial pathogens have been identified as causative factors of enteric diseases (Frydendahl, 2002; Jacobson et al., 2003; Aarestrup et al., 2008; Halaihel et al., 2010; Fairbrother and Gyles, 2012). However, it has also been shown that nutritional and management

factors also influence intestinal health in pigs (Callesen et al., 2007; Chase-Topping et al., 2007). Enterotoxigenic *Escherichia coli* (ETEC) that produce F4 or F18 adhesins are important pathogens often associated with post-weaning diarrhoea (PWD), which is characterised by diarrhoea or sudden death from 1 to 2 weeks post-weaning (Frydendahl, 2002). However, infection with ETEC can also be observed in older nursery pigs (Fairbrother and Gyles, 2012; Pedersen et al., 2014; Sato et al., 2016). There are also other bacterial causes of diarrhoea in pigs during the nursery period, such as *Lawsonia intracellularis* (LI) and *Brachyspira* spp., including *B. pilosicoli* (PILO) (Pedersen et al., 2014). For this reason, it is important that diagnostic procedures can identify the causal pathogens in such outbreaks.

ETEC is transmitted between pigs by the faecal-oral route, and it colonises the small intestine after attaching to receptors on the small intestinal epithelium using specific fimbrial adhesins. Production of enterotoxins causes a disturbance to the fluid balance in the small intestine, resulting in diarrhoea (Fairbrother et al., 2005). Proliferation of ETEC mainly takes place in the small intestine, and bacterial numbers remain constant from the ileum to the rectum, and rectal faecal samples are therefore useful for diagnosing ETEC-related diarrhoea (McAllister et al., 1979; Pedersen et al., 2010).

For many years the routine method for identifying ETEC-associated diarrhoea in weaned pigs, have been based on individual sampling of diarrhoeic pigs. Bacterial culture followed by either serotyping or detection of toxin (ST/LT) and fimbrial genes (F4/F18) by PCR of faecal samples collected from intestinal content or faeces is the most simple and effective way of providing a bacteriological diagnosis of the individual pig (Nagy and Fekete, 1999). Haemolytic activity of *E. coli* is a virulence marker (Frydendahl, 2002), and high concentrations of *E. coli* in a pure (or nearly pure) culture in the ileum is generally recognised as being indicative of colibacillosis (Francis, 1999). Recently, qPCR analysis of enteric pathogens in faecal samples collected by sock sampling from the floor of several pens has been described as a diagnostic tool for the simultaneous quantitative herd-level detection of different intestinal pathogens (Pedersen et al., 2015).

The aim of this study was to investigate whether bacterial culture and qPCR testing of faecal pen floor samples could be used as a diagnostic approach for identifying ETEC in groups of weaned pigs with clinical diarrhoea. The overall hypothesis was that the same *E. coli* pathotype could be detected in faecal samples from diarrhoeic nursery pigs and in samples taken from the floor of the pen where the pigs are housed.

The study was designed to evaluate the agreement at pen-level among three different diagnostic approaches for detecting ETEC in groups of diarrhoeic pigs:

D1. Bacterial culture and PCR testing for virulence genes; F4, F5, F6, F18, F41, STa, STb, LT and VT2e in selected *E. coli* colonies from three faecal samples per pen, obtained from individual pigs with clinical diarrhoea.

D2. Bacterial culture and PCR testing of virulence genes; F4, F5, F6, F18, F41, STa, STb, LT and VT2e in selected *E. coli* colonies from pen floor samples.

D3. qPCR testing for F4 and F18 genes in pen floor samples.

Furthermore, the value of using haemolytic activity as a virulence marker was evaluated.

2. Methods

2.1. Design

A cross-sectional study was performed in three commercial production herds in 2014. A total of 93 pigs in 31 different pens were sampled between 14 and 28 days post-weaning.

2.2. Inclusion of herds

The herds included in this study were previously selected for a clinical trial investigating batch medication for intestinal diseases in nursery pigs (Weber et al., 2017). The herds were characterised as high health herds (declared free of *Actinobacillus pleuropneumoniae* type 2, 6 and 12, porcine reproductive and respiratory syndrome virus, mange mites and lice (SPF-sus, 2015)), but with outbreaks of diarrhoea in nursery pigs requiring antibiotic treatment. The definition of an outbreak of diarrhoea requiring treatment has previously been characterised as ≥ 1.5 diarrhoeic pools per pen and a pen floor sample containing $\geq 35,000$ bacteria (calculated as *E. coli* F4 + *E. coli* F18 + LI + PILO)/g faeces (Pedersen et al., 2014). All herds had all-in all-out batch production in sectioned compartments with 2300 to 3600 pen places. The flooring consisted of one-third solid floor and two-thirds slatted floor. Feed was home-mixed and formulated with wheat, barley and soybean meal as the main ingredients, and fulfilled the Danish nutrient standards (Tybirk et al., 2015). The nursery pigs were crossbred Danav1 Yorkshire/Landrace and Duroc. All herds used 3000 ppm zinc oxide in the feed during the first 14 days post-weaning, which is common practice in Denmark.

2.3. Inclusion of pigs and pens

Eight pens were randomly selected within a batch, and 15 pigs from each of these pens were selected by systematic random sampling. If there were fewer than 15 pigs in the pen, all pigs were selected. Pigs in each herd were clinically examined on days 14, 21, and 28 post weaning. The diarrhoea status of the pigs was assessed by scoring faecal samples obtained by digital rectal manipulation. The faecal samples all were scored by one observer using a faecal consistency scale with four categories, where scores of 1 and 2 represented normal faeces and scores of 3 and 4 represented diarrhoea (Pedersen and Toft, 2011).

2.4. Sampling procedure

In pens with a diarrhoea prevalence of >25% among the sampled pigs, rectal samples from three diarrhoeic pigs and a pen floor sample were collected and stored in new, sealed plastic containers. The faecal pen floor sample was collected by wiping a gloved hand over the slatted floor. This sample was thoroughly stirred and the contents divided into two subsamples. The faecal samples from individual pigs and one of the pen floor samples were transported in a polystyrene box with freezing elements to the Laboratory for Pig Diseases in Kjellerup, Denmark for bacteriological examination by culture. The second pen floor sample was transported to the Danish National Veterinary Institute in Frederiksberg, Denmark for qPCR analyses.

2.5. Laboratory analyses

2.5.1. Bacteriology

An overview of sampling procedures is shown in Fig. 1. The pig and pen floor samples were aerobically cultured for *E. coli* by parallel culturing on Drigalski (an in-house selective and indicative medium for coliforms) and blood agar plates (Columbia agar (Oxoid) supplemented with 5% calf blood). The plates were incubated for 24 h at 37 °C. To identify the expected higher diversity of *E. coli* isolates in pen floor samples, a larger number of colonies were sampled from pen floor samples than pig samples. When possible, two haemolytic colonies surrounded by a clear zone of lysis and two non-haemolytic colonies were isolated from the pig samples, and where present, five haemolytic colonies and five non-haemolytic colonies were subcultured from the pen floor samples. In case of pure culture, only haemolytic or non-haemolytic were isolated.

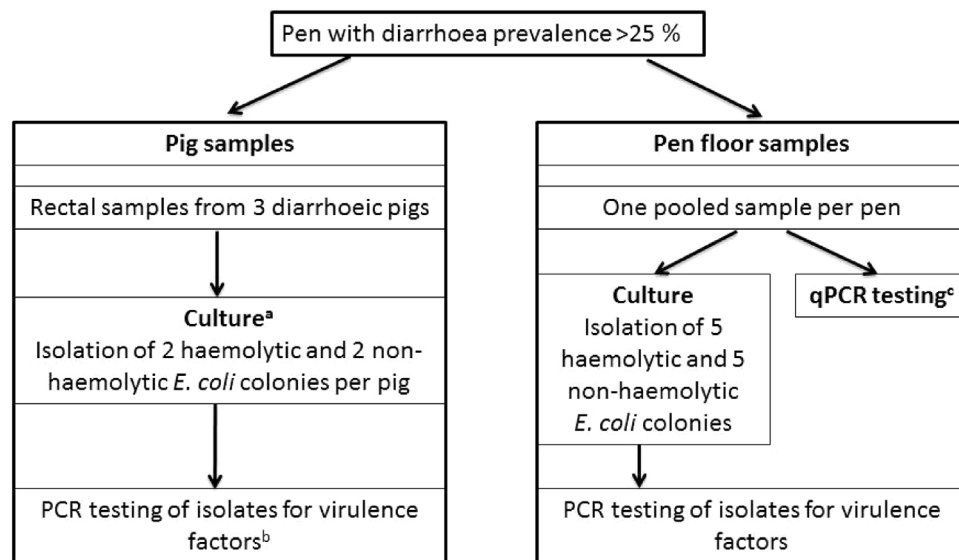


Fig. 1. Overview of sample procedure at pen level.

^aColonies surrounded by a zone of lysis after overnight growth at 37 °C on blood agar were classified as haemolytic, ^bdetection of virulence factor genes F4, F5, F6, F18, F41, STa, STb, LT and VT2e by PCR, ^cquantitate PCR for *E. coli* fimbrial gens F4, and F18, *Lawsonia intracellularis* and *Brachyspira pilosicoli*.

The isolates were analysed at the Danish National Veterinary Institute by real-time PCR for detection of virulence factor genes F4, F5, F6, F18, F41, STa, STb, LT and VT2e, as previously described by Frydendahl et al. (2001), with the exception that PCR cycling was performed on a Rotor-Gene Q (QIAGEN) PCR machine.

2.5.2. qPCR

Pen floor samples were thoroughly agitated and suspended in phosphate buffered saline (PBS) to obtain a 10% (w/v) faecal suspension. An aliquot of the suspension was transferred to a 2 ml microfuge tube and stored in a freezer at –20 °C until DNA extraction, as previously described by Pedersen et al. (2012). The DNA was subsequently stored in a freezer at –20 °C until the content of F4, F18, PILO and LI was quantified by qPCR, as previously described by Stahl et al. (2011), with the exception that standard curves for quantification were prepared from DNA extraction of spiked 10-fold dilution series using the same qPCR procedure as for the faecal specimens. The detection limits in bacteria or CFU per gram faeces were: 5.7×10^4 for F4, 1.5×10^3 for F18, and 2×10^3 for LI and PILO.

2.6. Statistics

All *E. coli* isolates were classified as ETEC when genes for one or more adhesin factors and one or more enterotoxins were detected. It was assumed that a pen contained ETEC-positive diarrhoeic pigs when the following criteria for the three diagnostic approaches were met:

D1. Minimum one pig in a pen should be ETEC-positive. An individual pig was classified as ETEC-positive when one or more ETEC isolates were demonstrated.

D2. A pen floor sample was classified as ETEC-positive when one or more ETEC isolates were demonstrated.

D3. A pen floor sample was classified as qPCR-positive when F4 and/or F18 were present in the sample.

Agreement among the three diagnostic approaches was evaluated by the calculation of observed agreement, and the statistical association was evaluated using a Fisher's exact test and Cohen's kappa coefficient.

3. Results

3.1. Data description

Pig and pen floor samples were collected from a total of 31 pens, including 5 pens in Herd 1, 17 pens in Herd 2 and 9 pens in Herd 3. The number of pigs per pen ranged from 12 to 40 with a mean of 29. Samples were taken from 10 pens at Day 14, 19 pens at Day 21 and 2 pens at Day 28 post-weaning. At the time of sampling, the mean pen-level diarrhoea prevalence of the sampled pigs was 31.6% (CL95% 27.9–35.3).

3.2. Bacteriology

E. coli colonies were isolated from 87 (93.5%) of the 93 sampled pigs, and in 27 (87.1%) of the 31 pen floor samples. An overgrowth of *proteus* was observed in four *E. coli*-negative samples which made it impossible to identify and isolate *E. coli* colonies. On six occasions, the sub-cultured isolates, selected from the dominating flora from the primary culture, was not identified as *E. coli*. Due to overgrowth of the dominating flora it was not possible to visually distinguish *E. coli* colonies from the dominating flora in these samples. A total of 208 *E. coli* isolates from pig faecal samples and 172 isolates from pen floor samples were used for further analyses. Haemolytic activity was detected on blood agar plates in 111 (29.2%) of the 380 colonies that were isolated. Pure (or nearly pure) cultures of haemolytic *E. coli* were observed in 13 (14.0%) of the 93 pig samples and in 4 (12.9%) of the 31 pen floor samples.

3.3. Virulence genes

The presence of virulence genes in the 380 *E. coli* isolates is shown in Table 1. The fimbrial gene F18 was detected in 95 (25.0%) isolates and was the only adhesin factor identified. Toxin genes were detected in 89 (93.7%) of the F18-positive isolates. The prevalence of toxin genes detected in F18-positive isolates were: STb (55.8%), STa (30.0%), LT (13.7%) and VT2e (2.4%). The most common virulence factor profile among the F18-positive isolates was: F18+STb+LT, found in 45 (11.8%), F18+STb found in 24 (6.3%), and F18+STa+STb found in 19 (5.0%) of the isolates. Toxin genes were detected in 149 (52.3%) isolates that were negative for fim-

Table 1Adhesin and toxin genes detected in 380 *E. coli* isolates from rectal samples of diarrhoeic pigs and pen floor samples 14–28 days post-weaning.

Sample type	Adhesin factor	No. of Isolates	Toxin profile							None
			LT	STa	STb	VT2e	STa,STb	STb,LT	STa,STb,VT2e	
Pig	F18+	46 (22.1%)	0	0	10	0	9	22	0	5
	None	162 (77.8%)	1	6	23	4	39	5	1	83
	Total	208 (100.0%)	1	6	33	4	48	27	1	88
Pen floor	F18+	49 (28.5%)	1	0	14	0	10	23	0	1
	None	123 (71.5%)	0	11	18	3	37	0	1	53
	Total	172 (100.0%)	1	11	32	3	47	23	1	54
Pig + pen floor	F18+	95 (25.0%)	1	0	24	0	19	45	0	6
	None	285 (75.0%)	1	17	41	7	76	5	2	136
	Total	380 (100.0%)	2	17	65	7	95	50	2	142

bial genes. A total of 136 (35.8%) isolates were negative for all of the analysed virulence factors. ETEC (fimbriae- and toxin-positive) isolates were detected in 22 (23.7%) of the 93 samples from pigs housed in 12 pens, and ETEC isolates were detected in 13 (41.9%) of the 31 pen floor samples.

3.4. qPCR analysis of pen floor samples

Results from the qPCR tests of 31 pen floor samples are shown in Table 2. LI genes were detected in 20 (64.5%) pen floor samples, F18 genes in 16 (51.6%) pen floor samples and PILO in 4 (12.9%) pen floor samples. F4 genes were only detected in 1 (3.2%) pen floor sample. The most common pathogen profiles found from qPCR testing of pen floor samples were: LI, which was found in 10 (32.3%) pens; F18 + LI found in 8 (25.8%) pens; F18 found in 5 (16.1%) pens.

3.5. Relationship between virulence factors and haemolytic activity

The relationship between pathogenicity, as represented by the presence of virulence factors (adhesin and toxin genes), and haemolytic activity is presented in Table 3. Haemolytic activity was strongly associated with the presence of virulence factors defining ETEC ($p < 0.001$). A sensitivity (SE) of 97.8% (CL95% = 92.1%–99.7%) and a specificity (SP) of 91.8% (CL95% = 88.0%–94.6%) were obtained when using haemolytic activity as a marker for the presence of ETEC virulence genes. Only two of the 269 isolates with non-haemolytic activity possessed ETEC virulence genes, corresponding to a high negative predictive value (NPV) of 99.3% (CL95% = 97.3%–99.9%). However, 24 of 111 isolates with haemolytic activity did not possess either adhesin or toxin genes, resulting in a positive predictive value (PPV) of 78.4% (CL95% = 69.6%–85.6%).

3.6. Relationship between detection of ETEC in pen floor and pig samples

A total of five comparisons were made among the different diagnostic approaches and presented in Table 4. The association between the detection of ETEC from pen floor samples by culture (D2) or by qPCR with a lower detection limit of 1.5×10^3 CFU per gram faeces (D3) and the detection of ETEC-positive diarrhoeic pigs by culture (D1) was statistically significant for D1 vs. D2 ($p < 0.001$), and for D1 vs. D3 ($p = 0.009$). The agreement between pen floor sample testing by bacterial culture or qPCR and the detection of ETEC-positive diarrhoeic pigs by culture was observed in 26 (D1 vs. D2: 83.9%, Kappa = 0.665) and 23 (D2 vs. D3: 74.2%, Kappa = 0.488) pens, respectively.

Disagreement was observed in pens with a low concentration of F18 genes in pen floor samples analysed by qPCR. Therefore an alternative cut-off (labelled AD2) for a positive qPCR sample was introduced by increasing the detection limit from 1.5×10^3 to 2×10^5 CFU per gram faeces:

AD3. A pen floor sample was classified as qPCR-positive when the concentration of F18 genes was above 2×10^5 CFU per gram faeces in the sample.

When applying this new alternative cut-off for qPCR-positive samples, agreement between qPCR testing of pen floor samples and the detection of ETEC-positive diarrhoeic pigs in the same pen increased to 27 (D1 vs. AD3: 87%, Kappa = 0.728) pens.

The relationship between the detection of ETEC by bacterial culture (D2) and qPCR in the same pen floor sample (D3) is shown in Table 4. The association between ETEC detection by bacterial culture and qPCR was statistically significant (D2 vs. D3: $p < 0.001$). Agreement between bacterial culture and qPCR of pen floor samples was observed in 26 (D2 vs. D3: 83.9%, Kappa = 0.679) pens. Applying the alternative cut-off value for the qPCR-positive samples (AD3) resulted in agreement in 28 (D2 vs. AD3: 90.3%, Kappa = 0.799) pens.

4. Discussion

In this study, faecal samples were collected from diarrhoeic pigs and pen floors 14, 21 and 28 days post-weaning in three Danish commercial pig farms. F18 and LI were the most frequently detected pathogens in the 31 pen floor samples tested by qPCR and the mean diarrhoea prevalence at pen level of the sampled pigs was 32.6%. Similar findings concerning pathogen profiles and diarrhoea prevalence have previously been reported from outbreaks of diarrhoea in flocks of Danish nursery pigs (Pedersen et al., 2015). Pure (or nearly pure) cultures of haemolytic *E. coli* were observed in 14% of the diarrhoeic pigs, indicating that, in a large proportion of the pigs, *E. coli* was not the primary pathogen. Furthermore, qPCR results from the pen floor samples showed that *L. intracellularis* were frequently present and could therefore be the causative agent of some of the clinical diarrhoea observed in this study.

Haemolytic activity was found to be a useful marker of ETEC, as demonstrated by virulence genes with a sensitivity of 97.8% and a specificity of 91.8%, which is similar to previous published results from PWD pigs (Frydendahl, 2002; Do et al., 2005; Chapman et al., 2006; Zhang et al., 2007). The dominant fimbrial gene that was found was F18, which was detected in 25% of the isolates. In previous studies of isolates from PWD cases, F4 and F18 were the most frequently detected fimbrial genes, with a reported prevalence ranging from 22.9% to 64.6% for F4 and 12.9% to 46.9% for F18 (Frydendahl, 2002; Chapman et al., 2006; Zhang et al., 2007; Zajacova et al., 2012; Sato et al., 2016). The lack of F4 fimbrial genes

Table 2Quantitate PCR results for *E. coli* fimbrial genes F4 and F18, *Lawsonia Intracellularis* and *Brachyspira Pilosicoli* from 31 pen floor samples.

Pen	Bacteria/g faeces				Sum
	F4	F18	<i>Lawsonia Intracellularis</i>	<i>Brachyspira Pilosicoli</i>	
1	0	564,000	1,370,000	0	1,426,400
2	0	239,000	3,030,000	0	3,269,000
3	0	924,000	1250	0	925,250
4	0	5,430,000	0	0	5,430,000
5	0	666,000	0	0	666,000
6	0	498,000	0	0	498,000
7	0	0	59,700	0	59,700
8	0	0	338,000	0	338,000
9	0	0	7730	0	7730
10	0	138,000	1640	0	139,640
11	0	0	0	0	0
12	0	27,200	10,300	0	37,500
13	0	0	8230	0	8230
14	0	21,300	0	6070	27,370
15	0	0	33,300,000	0	33,300,000
16	0	0	1,110,000	0	1,110,000
17	0	536,000	0	0	536,000
18	0	427,000	0	0	427,000
19	0	0	0	0	0
20	0	0	2550	0	2550
21	0	56,400	1,300,000	0	1,356,400
22	0	922,000	2400	0	924,400
23	0	0	0	8320	8320
24	0	0	0	0	0
25	0	0	9,740,000	0	9,740,000
26	0	0	3,410,000	0	3,410,000
27	0	0	43,000	10,800	53,800
28	0	0	18,400	0	18,400
29	0	1,300,000	1260	968	1,302,228
30	0	669,000	992	0	669,992
31	260,000	14,100,000	1,370,000	0	14,360,000
Total positive pens	1	16	20	4	

Table 3Relationship between presence of virulence factor genes and haemolysis in *E. coli* isolates.

Virulence factors ^a					Parameter	Estimate (95% CI)
Haemolytic activity ^b		+	–	Total	Sensitivity	97.8% (92.1%–99.7%)
	+	87	24	111	Specificity	91.8% (88.0%–94.6%)
	–	2	267	269	Positive predictive value	78.4% (69.6%–85.6%)
	Total	89	291	380	Negative predictive value	99.3% (97.3%–99.9%)

^a Isolates harbouring both adhesin and toxin genes were assigned '+', all other were assigned '–'.^b Colonies surrounded by a zone of lysis after overnight growth at 37 °C on blood agar were assigned '+'.

in the current study could be coincidental because samples were acquired from only three herds, or because the sampled diarrhoeic pigs in the current study were older than pigs in the previous studies. In the study farms, high levels of zinc oxide was added in the feed during the first two weeks post weaning which could have resulted in delayed outbreaks of diarrhoea and could thereby favour infections with F18 rather than F4 ETEC. Another possible explanation is that all pigs from the study farms were obtained from the Danavl breeding company. In 2002, resistance genes against F4 receptors were identified in pigs (Jorgensen et al., 2003) and Danavl have implemented selective breeding for F4 resistance since 2003.

The association between clinical disease and fimbriae detection is not evident. In a study from Australia, F18 fimbriae genes were only detected in diarrhoeic pigs and not in healthy pigs (Chapman et al., 2006). In contrast, it has also been demonstrated that F18-positive ETEC can be detected in healthy pigs with no signs of diarrhoea (Schierack et al., 2006; Weber et al., 2015).

Of the F18-positive isolates, 93.7% were toxin-positive, indicating that detection of fimbrial genes is a good predictor of virulence.

In contrast, detection of toxins as a sole predictor of pathogenicity proved to be problematic. Approximately half of all analysed isolates possessed toxin genes but were negative for fimbrial genes and therefore not considered ETEC. In this study, only five of the most common adhesin factors (F4, F5, F6, F18 and F41) were included in the analysis. Other adhesin factors, known to contribute to the virulence of ETEC in nursery pigs (such as intimin and AIDA) were not included in the analysis and could potentially have influenced the conclusions drawn from the study (Frydendahl, 2002; Fairbrother and Gyles, 2012). Furthermore other still unknown adhesin factors could also be involved in the pathogenesis of the diseased pigs.

The sampling procedure in this study was based on isolation of *E. coli* colonies with or without haemolytic activity after culture of faecal samples. Depending on presence after culture, we examined a maximum of two isolates with haemolytic activity and two non-haemolytic isolates from three diarrhoeic pigs per pen, in order to establish the ETEC status of all the pigs within the same pen. Furthermore, we examined a maximum of five haemolytic,

Table 4

Comparison of diagnostic approaches for detecting ETEC in pen floor and pig faecal samples by bacterial culture and PCR or quantitative PCR.

Comparison of diagnostic approaches	p-value ^e	Observed agreement (Pens with agreement/total pens)	Kappa ^f (Standard Error)
D1 ^a vs. D2 ^b	<0.001	0.839 (26/31)	0.665 (0.179)
D1 vs. D3 ^c	0.009	0.742 (23/31)	0.488 (0.174)
D1 vs. AD3 ^d	<0.001	0.871 (27/31)	0.728 (0.180)
D2 vs. D3	<0.001	0.839 (26/31)	0.679 (0.176)
D2 vs. AD3	<0.001	0.903 (28/31)	0.799 (0.179)

^a D1: pig samples analysed by culture and real-time PCR were classified as ETEC-positive when one or more of the four potential isolates from each pig in a pen were harbouring both adhesin and toxin genes.

^b D2: Pen floor samples analysed by culture and real-time PCR were classified as ETEC-positive when one or more of the ten potential isolates from each pen were harbouring both adhesin and toxin genes.

^c D3: pen floor samples analysed by qPCR were considered positive for fimbrial F18 genes when the amount of bacteria per gram faeces was above the detection limits (1.5×10^3 CFU per gram faeces).

^d AD3: pen floor samples analysed by qPCR were considered positive for fimbrial F18 genes when the detection of bacteria per gram faeces was above 2×10^5 CFU per gram faeces.

^e Fisher's exact test.

^f Cohen's kappa coefficient significance value <0.05.

and five non-haemolytic isolates per pen floor sample to establish the ETEC status of the sample. If the sample size (i.e. pigs per pen and examined colonies per sample) had been increased, it is likely that the sensitivity of these diagnostic approaches to detect ETEC-positive pigs would also increase. The selected sample size was chosen partly for convenience and economic factors related to laboratory analysis, as well as to simulate the commonly used diagnostic approach of Danish veterinary practitioners.

This study demonstrates that it is possible to detect the same ETEC pathotypes from individual pig samples and pen floor samples. The results show that pen floor samples might be a useful diagnostic approach for detecting ETEC-positive pigs that can be applied in veterinary practice. Using direct qPCR testing of faecal samples for diagnosing ETEC have the advantages of easy handling of samples and a short response time for test results, whereas bacterial culture is a cheap diagnostic method which can be performed without advanced laboratory equipment. There was a good agreement between the detection of virulent *E. coli* isolates from both diarrhoeic pigs and from pen floor samples when using bacterial culture and subsequent demonstration of virulence genes in selected *E. coli* colonies. It is likely that the lower agreement observed when using qPCR detection of fimbrial genes in pen floor samples is due to the qPCR analysis being more sensitive than bacterial culture from three diarrhoeic pigs. This is further supported by the higher agreement observed when the lower detection limit of the qPCR analysis was raised to 2×10^5 CFU per gram faeces.

The study also revealed a good agreement when comparing qPCR detection of fimbrial genes to demonstration of virulence genes following bacterial culture of selected *E. coli* colonies in pen floor samples. This finding indicates that the qPCR analysis used in this study is appropriate for detecting F18 fimbrial genes from pen floor samples where virulent F18-positive *E. coli* bacteria are present.

The prudent use of antimicrobials in production animals is a focus point throughout the world (European-Commission, 2015; OIE, 2016). In veterinary practice, this involves making the right decision regarding the initiation of antimicrobial treatment with the aim of achieving the most efficient treatment of diseased animals while maintaining the lowest possible antibiotic consumption.

This study suggests a new diagnostic approach for detecting ETEC-positive nursery pigs by examining the pen floor rather than faecal samples from diarrhoeic pigs. Recently, sock samples have been described as an effective way of obtaining samples from pen floors (Pedersen et al., 2015). Applying qPCR testing to faecal samples has the advantage of including F4/F18 ETEC, LI and PILO simultaneously. These new diagnostic approaches can improve the diagnosis of enteric diseases in nursery pigs and thereby help to achieve the goal of a more prudent use of antibiotics in pig production. Direct testing of pen floor samples for multiple enteric pathogens can give a fast and precise diagnosis, supporting the decision for initiation of treatment and antimicrobial compound selection. The diagnostic approaches suggested in this study should be further evaluated under field conditions to confirm the results. Ideally this study should be repeated in herds dealing with PWD 1–2 weeks post weaning where F4 and F18 ETEC would be considered as primary pathogens. Future research on the value of resistance profiling of ETEC isolated from pen floor samples is recommended in order to assist in the selection of narrow-spectrum antibiotics for treatment of diarrhoea caused by ETEC.

5. Conclusions

This study showed that both bacterial culture and qPCR testing of pen floor samples can be used as a diagnostic approach for detecting groups of ETEC-positive diarrhoeic nursery pigs. An acceptable agreement for the detection of ETEC-positive diarrhoeic nursery pigs in pen samples was observed for both the bacterial culture of pen floor samples and qPCR. Furthermore, haemolytic activity was a useful marker for isolates containing both adhesins and virulence genes.

Competing interests

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of this paper.

Authors' contributions

KP, SH and NW designed the sampling protocol and selected methods; SH conducted the bacteriology; CH conducted the qPCR analyses; NW performed the data sampling; NW performed the statistical analysis; JPN, CFH, KP, SH, SEJ, CH and NW devised the study and drafted the manuscript. All authors contributed to finalising the manuscript. All authors read and approved the final manuscript.

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6.4 Paper IV

Comparison of antimicrobial resistance in *E. coli* isolated from rectal and floor samples in pens with diarrhoeic nursery pigs

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Abstract

The prudent use of antibiotics in veterinary medicine involves selecting antibiotic compounds with narrow-spectrums targeted against the specific pathogens involved. The same pathotype of enterotoxigenic *E. coli* (ETEC) was recently found both in diarrhoeic pigs and in samples from the pen floor where the pigs were housed. The aim of this study was to evaluate the agreement between antibiotic resistance testing of ETEC isolates from pen floor and pig samples.

Across three Danish pig herds, faecal samples were collected from the floors of 31 pens that had a within-pen diarrhoea prevalence of >25%, and from rectal samples of 93 diarrhoeic nursery pigs from the same pens. A total of 380 *E. coli* isolates were analysed by PCR and classified as ETEC when genes for adhesin factors and enterotoxins were detected. Minimum inhibitory concentrations of 13 antimicrobial agents were determined by the broth microdilution method. Isolates were classified as resistant based on clinical breakpoints.

The rate of resistance in ETEC isolates were as follows: sulphamethoxazole (50.3%), ampicillin (45.5%), trimethoprim (44.9%), streptomycin (42.4%), tetracycline (36.1%), spectinomycin (20.5%), gentamicin (3.4%), neomycin (0.5%) and amoxicillin+clavulanat acid (0.2%), ciprofloxacin (0.0%), colistin (0.0%), and ceftiofur (0.0%). When comparing resistance profiles from isolates from the same pen (pen floor and pig samples), excellent agreement was observed for all antimicrobial agents.

The findings of this study indicate that ETEC isolates from the pen floor could provide a convenient method of resistance testing and selecting clinically relevant antimicrobial agents for the treatment of diarrhoeic pigs.

Keywords: Antimicrobial use, resistance, pen floor samples, diarrhoea, ETEC, nursery pigs

Introduction

The risk of antimicrobial resistance (AMR) spreading from food-producing animals to humans is a major concern that attracts considerable political attention. The World Health Organisation (WHO) has identified AMR as a global threat to human health, and asserted that action to combat AMR must be taken to avoid a post-antibiotic era (WHO, 2014). The prudent use of antimicrobials for production animals is therefore a focus point throughout the world (European-Commission, 2015; OIE, 2016). Prudent use is defined as the choice of antimicrobials based on combined information from clinical experience, the expected susceptibility of the target pathogen, the route of administration, expected activity at the site of infection and the epidemiological history of the production unit, in particular previous AMR profiles (OIE, 2016). By using AMR profiles, veterinarians are able to select antimicrobial compounds with the narrowest spectrum of activity sufficient to target the pathogen (European-Commission, 2015). Good veterinary practice involves diagnostic tools to assist in making the right decision about when to initiate antimicrobial treatment, with the aim of achieving the most efficient treatment of diseased animals with the lowest possible antibiotic consumption. Previous published results from our group have shown that faecal pen floor samples can be used to diagnose enteric diseases from groups of pigs (Pedersen et al., 2015; Weber et al., 2017). Furthermore, in outbreaks of ETEC-induced diarrhoea, the same pathotype of ETEC was demonstrated in rectal faecal samples from diarrhoeic pigs and in faecal samples from the pen floor where the pigs were housed (Weber et al., submitted). We therefore hypothesise that using ETEC isolated from pen floor samples could be a convenient and relevant method for resistance testing and selection of antimicrobial agents.

The aim of this study was to investigate resistance profiles in ETEC and Non-ETEC isolates and to evaluate whether ETEC isolates from faecal pen floor samples could be used for resistance

profiling. This was achieved by comparing resistance profiles in ETEC isolates from pen floor samples to faecal samples obtained per rectum from individual pigs in the same pens. Resistance profiling of pathogenic *E. coli* is highly relevant in veterinary practice when choosing the type of antimicrobial agent for treatment.

The first objective of the study was to evaluate the agreement between resistance profiles of ETEC isolated from pen floor samples and from individual rectal samples from pigs.

The second objective was to compare resistance profiles from ETEC isolates and Non-ETEC isolates.

Method

Design

A cross-sectional study was performed in three commercial production herds in 2014. A total of 31 pens were selected and 93 pigs from these pens were sampled 14 to 28 days after weaning.

Herd description

A thorough description of the herds included in the study is published in Weber et al. (2017).

The herds were previously selected for a clinical trial investigating batch medication for intestinal diseases in nursery pigs. In brief, the herds were characterised as high-health herds declared free of *Actinobacillus pleuropneumoniae* type 2, 6 and 12, porcine reproductive and respiratory syndrome virus, mange mites and lice (SPF-sus, 2015), but with outbreaks of diarrhoea in nursery pigs requiring antimicrobial treatment (Pedersen et al., 2014). All herds had all-in all-out batch production by compartment and the flooring consisted of 1/3 solid floor and 2/3 slatted floor. Feed was home-mixed and formulated with wheat, barley and soybean meal as the main ingredients, and fulfilled the Danish nutrient standards (Tybirk et al., 2015). The nursery pigs were DanAvl crossbreeds between Yorkshire/Landrace and Duroc. All herds used 3,000 ppm zinc oxide in the feed during the first 14 days after weaning.

Sampling procedure

In pens with a diarrhoea prevalence of 25% or above, rectal samples from three diarrhoeic pigs and a faecal pen floor sample were collected and stored in sealed plastic containers. A diarrhoeic pig was identified by scoring the rectal sample using a faecal consistency scale with four categories, where scores of 1 and 2 represented normal faeces and scores of 3 and 4 represented diarrhoea (Pedersen and Toft, 2011). The pen floor samples were collected by running a gloved hand over the slatted floor. The cooled faecal samples were transported for bacteriology in a polystyrene box with freezing elements to the Laboratory for Pig Diseases in Kjellerup, Denmark.

Bacteriology

The pig and pen floor samples were aerobically cultured for *E. coli*. Parallel culturing was performed on Drigalski (in-house selective and indicative medium for coliforms) and blood agar plates (Columbia agar (Oxoid) supplemented with 5% calf blood). Plates were incubated for 24 hours at 37°C. After culture, two coliform colonies with haemolytic activity (if present) and two coliform colonies with non-haemolytic activity were isolated from each sample. Haemolytic isolates were defined as colonies surrounded by a zone of lysis. If present, five coliform colonies with haemolytic activity and five coliform colonies with non-haemolytic activity were isolated from the pen floor samples. The selected isolates were analysed at the Danish Veterinary Institute using the 5'-nuclease assay (TaqMan PCR) previously described for the detection of virulence factor genes: F4, F5, F6, F18, F41, STa, STb, LT and VT2e (Frydendahl et al., 2001).

Antimicrobial susceptibility testing

The antimicrobial concentration ranges and clinical breakpoints of 13 antimicrobial agents included in the panel are shown in Table 1. The panel comprises clinically relevant antimicrobial agents for the treatment of porcine *E. coli* infections, in agreement with international guidelines (Burch et al., 2008; DANMAP, 2013). Minimum inhibitory concentrations (MIC) were

determined by the broth microdilution method in 96-well microtitre plates using the Sensititre system (Thermo Fisher Scientific, Waltham, Massachusetts, USA), as described in the standards manual of the Clinical and Laboratory Standards Institute (CLSI, 2013). The *E. coli* reference strain ATCC 25922 was used as a control organism. The plates were incubated for 20 hours at 37°C in an aerobic atmosphere. The MIC was defined as the lowest concentration producing no visible growth. Clinical breakpoints derived from The European Committee on Antimicrobial Susceptibility Testing (EUCAST) or CLSI were used to interpret MIC values (CLSI, 2013; DANMAP, 2010; EUCAST, 2016).

Statistical analysis

Comparisons of resistance are presented in the tables. Herd-level comparisons were made between ETEC and Non-ETEC isolates. All other comparisons performed in this study were done on ETEC isolates. Statistical analyses were performed in R version 3.1.2 (R-Core-Team, 2014), with mixed models implemented using the lme4 package (Bates et al., 2015). Herd, batch and sample were used as random effects in all the statistical models to account for clustering at herd, batch and sample level. To estimate the effect of ETEC status on resistance, a generalised linear model was used for each antimicrobial agent, with resistance as outcome and ETEC status as primary explanatory variable. A linear mixed model was used to describe multiple resistance, with total counts of antimicrobial agent resistance per isolate (0-13) as the primary outcome, and ETEC status as the primary explanatory variable.

Results

Resistance in ETEC and Non-ETEC isolates

A total of 380 *E. coli* isolates were obtained and used for further analysis. PCR testing for STa, STb, LT and VT2e toxin and F4, F5, F6, F18, F41 fimbriae genes revealed 89 isolates classified as ETEC, and 291 as Non-ETEC. Table 2 shows the distribution of MIC and the rate of resistance of isolates to the 13 antimicrobial agents used in this study. The overall rate of

resistance was above 1% in seven antimicrobial agents: sulphamethoxazole (50.3%), ampicillin (45.5%), trimethoprim (44.9%), streptomycin (42.4%), tetracycline (36.1%), spectinomycin (20.5%) and gentamicin (3.4%). Low resistance rates were observed in neomycin (0.5%) and amoxicillin+clavulanic acid (0.2%). Full sensitivity was observed for ciprofloxacin, colistin, and ceftiofur. The odds of an isolate being resistant were significantly higher ($p<0.05$) in ETEC isolates compared to Non-ETEC isolates for ampicillin, apramycin, gentamicin, sulphamethoxazole, tetracycline and trimethoprim. Overall, significantly more ($p<0.001$) Non-ETEC isolates were fully sensitive to all tested antimicrobial agents (37.8%) compared to ETEC isolates (7.9%). On average, ETEC isolates were resistant to 3.3 antimicrobial agents, significantly higher ($p<0.001$) than Non-ETEC isolates, which were on average resistant to 2.2 antimicrobial agents.

Resistance distribution at herd level

The distribution of resistant ETEC and Non-ETEC isolates at herd level is shown in Table 3. In ETEC isolates from Herd 1, resistance was observed in eight different antimicrobial agents, compared to one and four antimicrobial agents in isolates from Herds 2 and Herd 3, respectively. Resistance to ampicillin was observed in isolates from all three herds. Resistance against sulphamethoxazole, trimethoprim and streptomycin was observed in Herds 1 and 3. Resistance against apramycin, gentamicin, spectinomycin and tetracycline was only observed in Herd 1. The herd-level distribution of resistance for Non-ETEC isolates was more similar than for ETEC isolates. Resistance against sulphamethoxazole, spectinomycin, streptomycin, tetracycline, and trimethoprim was present in all three herds and accounted for the majority of resistance in Non-ETEC isolates.

Antimicrobial resistance profiles

Table 4 shows the 28 different AMR patterns observed among the isolates. The ETEC isolates were clustered in fewer patterns (9) than the Non-ETEC isolates (22), and four patterns (17, 13,

10 and 1) were observed in both Non-ETEC and ETEC isolates. According to the European Centre for Disease Prevention and Control, multidrug resistance (MDR) is defined as resistance to ≥ 3 classes of antimicrobials (Magiorakos et al., 2012). MDR was observed in 62 (69.7%) ETEC isolates, which was a significantly higher ($p < 0.001$) percentage than the 144 (49.5%) Non-ETEC isolates.

Comparison of resistance patterns in ETEC isolates

Table 5 shows the resistance patterns of ETEC isolates from pig and pen floor samples. In 10 pens, ETEC isolates were demonstrated in both pig samples and in the pen floor samples simultaneously. Within-pen variation in resistance patterns was observed in both pig and pen floor isolates. Overall, there was no overlap of resistance patterns between the three study herds. Good agreement was observed when comparing resistance patterns between pig and pen floor isolates. The same resistance patterns were observed in pig isolates and corresponding pen floor isolates in 7 of the 10 pens.

Comparison of resistance to selected antimicrobial agents

In the following sections, resistance classifications of ETEC isolates were compared for selected antimicrobial agents that had an overall resistance rate of $>1\%$.

Within-sample agreement

Table 6 shows the agreement in the resistance classifications for selected antimicrobial agents between ETEC isolates obtained from the same sample. In this study, it was possible to make 18 comparisons of resistance between two isolates from the same pig sample. Overall, good agreement of resistance was observed between isolates obtained from the same pig. Between 0 and 5 ETEC were isolated per pen floor sample. It was possible to make a comparison between multiple isolates from 11 pen floor samples. As with the pig samples, a good agreement was observed between isolates from the same pen floor sample. Some

disagreement was observed for streptomycin and spectinomycin resistance, where only 6 and 8 (of 11) pen samples showed agreement, respectively.

Agreement between pigs within pen

Within each pen, 1 to 3 diarrhoeic pigs were sampled. A pig was classified as resistance positive for a specific antimicrobial agent if a minimum of one ETEC isolate from the pig was found to be resistant. In 7 pens, ETEC was detected in more than one diarrhoeic pig. When comparing the resistance classification in these 7 pens, perfect agreement between pigs from the same pen was observed for apramycin, gentamicin, spectinomycin and tetracycline resistance. Agreement for ampicillin, streptomycin, sulphamethoxazole and trimethoprim resistance was observed in 6 of 7 pens.

Agreement between pig and pen floor isolates

When comparing resistance in pig isolates and in pen floor isolates from the same pen, the following definition of resistance classification was used:

Pig isolate resistance: The pigs were classified as resistance positive for a specific antimicrobial agent if one or more ETEC isolates from one or more pigs in the pen were resistant.

Pen floor resistance: A pen floor sample was classified as resistance positive for a specific antimicrobial class if one or more ETEC isolates from the sample were resistant.

It was possible to make a comparison of resistance classification between pig isolates and the corresponding pen floor samples in 10 pens. By using the previously mentioned definitions, perfect agreement was observed in ampicillin, apramycin, gentamicin, sulphamethoxazole, tetracycline and trimethoprim resistance, whereas agreement in spectinomycin and streptomycin resistance was observed in 9 and 7 pens, respectively.

Discussion

This study investigated resistance in *E. coli* isolates from pig and pen floor samples. The isolates classified as Non-ETEC can be regarded as indicator bacteria, whereas ETEC isolates are considered clinical isolates. Indicator bacteria are ubiquitous and present as commensals in both animal and human reservoirs, and can be monitored to detect the occurrence of AMR in different reservoirs throughout the food chain (DANMAP, 2016).

Overall, full susceptibility was observed in the two antibiotic agents ciprofloxacin and ceftiofur, classified by the WHO as critically important antimicrobials for human medicine (WHO, 2012). These findings correspond well with the use of flouroquinolones in pigs being strictly limited in Denmark since 2002, and the voluntary ban on the use of third- and fourth-generation cephalosporins in the Danish pig industry since 2010 (DANMAP, 2016). Furthermore, full susceptibility was observed to colistin, which has recently been classified as a critically important antimicrobial for the treatment of carbapenemase-resistant infections in human medicine (DANMAP, 2016). Resistance in Non-ETEC isolates from this study were observed for the same antimicrobial agents and with similar rates to those previously reported in indicator *E. coli* from Danish resistance surveillance (DANMAP, 2014). Furthermore, little variation in Non-ETEC resistance was observed at herd level. This indicates that the resistance found in the three farms from this study is representative of Danish pig farms in general, and that background resistance against the same antimicrobial agents is present. However, a higher between-herd variation was reported in studies of AMR among faecal indicator *E. coli* from North America (Bunner et al., 2007; Dunlop et al., 1998; Rosengren et al., 2008). A possible reason that comparatively little variation was observed in our study could be that factors influencing AMR (such as antimicrobial pressure, movement and flow of humans and animals, or interaction with rodents) were similar in the three herds.

The highest overall resistance rate in ETEC isolates from this study was observed for ampicillin, sulphamethoxazole, tetracycline and trimethoprim. Similar findings have been reported for clinical isolates from diarrhoeic nursery pigs submitted to diagnostic laboratories in Denmark (DANMAP, 2013; Hendriksen et al., 2008).

The between-herd variation in resistance for ETEC isolates was markedly more diverse than for Non-ETEC isolates. This may be due to a more clonal distribution of virulent strains, and emphasises the importance of performing antimicrobial susceptibility testing at farm level when selecting antimicrobial agents for treatment of *E. coli*-related diarrhoea. Furthermore, susceptibility testing should always be performed on ETEC rather than Non-ETEC isolates, since the resistance profiles may differ between herds.

In this study, ETEC isolates were more resistant than Non-ETEC isolates, which indicates that AMR may be genetically linked to virulence factor genes. This finding has previously been described by Sato et al. (2015), who observed a strong association between fimbriae and toxin genes and AMR in 185 *E. coli* isolates from diarrhoeic pigs in Brazil. The same pattern of a higher resistance rate in clinical isolates compared to indicator isolates has been observed in Denmark for many years (DANMAP, 2013). The adverse consequences of more resistance in clinical isolates underline the importance of prudent antimicrobial use in the treatment of ETEC-related diarrhoea in pigs. To prevent resistance from developing, the relevant susceptibility testing should be considered when selecting the antimicrobial agents to be used for treatment. Treatment of healthy pigs should be avoided to ensure the effect of antimicrobial agents on clinical isolates.

This study showed that the resistance patterns in ETEC isolates were more homogeneous than in Non-ETEC isolates. A possible explanation for this finding is that the ratio of ETEC/Non-ETEC isolates in this study was 3 to 1. Alternatively, it could be due to the clonal distribution of

virulent strains previously described and supported by the large between-herd variation in resistance, which demonstrates that different clones of ETEC isolates predominate among different herds.

Several comparisons of resistance in ETEC isolates were performed in this study. The goal of these comparisons was to identify a convenient and representative sampling method that would provide the most precise susceptibility testing of ETEC isolates. Overall, good agreement was observed in all of the comparisons performed. The results show that no extra information on resistance is gained when multiple isolates are tested, regardless of whether the sampling is performed on isolates from diarrhoeic pigs or pen floor isolates. To our knowledge, there is no previously published report of the within-sample variation in resistance in clinical *E. coli* isolates. Publications on the variation in resistance have mainly focussed on national resistance surveillance, where the resistance of indicator *E. coli* isolated from healthy pigs has been examined (Yamamoto et al., 2014). However, low within-sample variation in resistance was reported in a Norwegian study of *E. coli* isolates from clinically healthy pigs (Brun et al., 2002). All isolates from the current study were classified as resistant or sensitive based on MIC values above or below the clinical breakpoints determined by EUCAST or CLSI. The greatest level of disagreement in resistance status in the within-sample comparisons and comparisons between pig and pen floor isolates was observed for spectinomycin and streptomycin. The reason for this observed disagreement could be that the MIC values for these antimicrobials were clustered around the breakpoints, making a single dilution step sufficient to change the isolate from susceptible to resistant. The results concerning resistance to spectinomycin and streptomycin must therefore be interpreted with caution due to the uncertainty of the true susceptibility status. The comparison of resistance between pig isolates and pen floor isolates from the same pen revealed good agreement. Together with the recent finding of similar ETEC isolates with the

same virulence profiles in diarrhoeic pig samples and in samples from the pen, a new diagnostic approach based on pen floor samples has been suggested (Weber et al., submitted). This may be combined with susceptibility testing of the same isolates, as demonstrated in the present study. Conclusions in this study were based on sampling ETEC isolates from diarrhoeic pigs 14 to 28 days post-weaning in three herds.

To confirm the results, this study should be further evaluated under field conditions in additional herds dealing with colibacillosis 1 to 2 weeks post-weaning, where ETEC isolates would be considered primary pathogens.

Conclusion

This study indicates that resistance testing of ETEC isolates from pen floor samples can be used as a convenient sampling method for resistance testing and in the selection of clinically relevant antimicrobial agents in the treatment of diarrhoeic pigs. The herd-level variation in resistance within ETEC isolates emphasises the importance of performing antimicrobial susceptibility testing at farm level when selecting antimicrobial agents for the treatment of *E. coli*-related diarrhoea.

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Conflict of interest

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of this paper.

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Table 1: Antibiotic concentration ranges and resistance breakpoints used for susceptibility testing of *E. coli* (n = 380) isolated from faecal samples from weaned pigs and pen floors

Antimicrobial class	Antimicrobial agent	Abbreviations	Concentration used (µg/ml)	Clinical breakpoint (µg/ml) *
Penicillins	Ampicillin	AMP	1 - 32	≥32 ^a
B-Lactam/β-lactamase inhibitors	Amoxicillin/clavulanic acid	AUC	2/1 - 32/16	≥32/16 ^a
Folate pathway inhibitors	Trimethoprim	TMP	1 - 32	≥16 ^b
	Sulphamethoxazole	SMX	64 – 1,024	≥512 ^a
	Gentamicin	GEN	0.5 - 32	≥16 ^a
	Apramycin	APR	4 - 32	≥16 ^b
Aminoglycosides	Streptomycin	STR	8 - 128	≥32 ^b
	Spectinomycin	SPE	16 - 256	≥128 ^b
	Neomycin	NEO	2 - 32	≥16 ^b
	Ciprofloxacin	CIP	0.015 - 4	≥4 ^c
Quinolones	Ceftiofur	XNL	0.5 - 8	≥8 ^d
Cephalosporins	Tetracycline	TET	2 - 32	≥16 ^a
Tetracyclines	Colistin	COL	1 - 16	≥16 ^b
Polymyxins				

^a CLSI-approved breakpoints based on human data. ^b Breakpoints routinely used by the Laboratory of Swine Diseases, Kjellerup, Denmark and by the Danish Veterinary Institute, Frederiksberg, Denmark. ^c CLSI-approved breakpoint for Enrofloxacin based on dog data and considered to be representative of Ciprofloxacin. ^d CLSI-approved breakpoint based on cattle data.

Paper IV

Table 2: Distribution of MICs and occurrence of resistance in 89 ETEC and 291 Non-ETEC isolates

Antimicrobial agent	Isolate type	% resistant	95% CI*	Distribution of MICs																																					
				0.01	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048																			
Amoxicillin+	ETEC	0.0 ^a	0.0, 4.1																																						
Clavulanic acid**	Non-ETEC	0.3 ^a	0.0, 1.9																																						
Ampicillin	ETEC	60.7 ^a	49.7, 70.8																											12	23					54					
	Non-ETEC	40.9 ^b	35.2, 46.8																											49	96	23	4			119					
Apramycin	ETEC	14.6 ^a	8.0, 23.7																													74	2			13					
	Non-ETEC	0.7 ^b	0.1, 2.5																													250	39	2							
Ceftiofur	ETEC	0.0 ^a	0.0, 4.1																										89												
	Non-ETEC	0.0 ^a	0.0, 1.3																										287	3	1										
Ciprofloxacin	ETEC	0.0 ^a	0.0, 4.1																				23	24	42																
	Non-ETEC	0.0 ^a	0.0, 1.3																				56	73	155	3	1														
Colistin	ETEC	0.0 ^a	0.0, 4.1																											58	10	20	1								
	Non-ETEC	0.0 ^a	0.0, 1.3																											288	3										
Gentamicin	ETEC	14.6 ^a	8.0, 23.7																										59	17				8	5						
	Non-ETEC	0.0 ^b	0.0, 1.3																										167	112	12										
Neomycin	ETEC	0.0 ^a	0.0, 4.1																												85	4									
	Non-ETEC	0.7 ^a	0.1, 2.5																												279	10			2						
Spectinomycin	ETEC	18.0 ^a	10.6, 27.6																															48	2	23	3	1	12		
	Non-ETEC	21.3 ^a	16.7, 26.5																															175	33	21	9	17	36		
Streptomycin	ETEC	29.2 ^a	20.1, 39.8																														45	13	5		9	17			
	Non-ETEC	41.9 ^a	36.2, 47.8																														147	14	8	20	22	80			
Sulphamethoxazole	ETEC	69.7 ^a	59.0, 79.0														27				62																				
	Non-ETEC	44.7 ^b	24.0, 7.5														161	1			129																				
Tetracycline	ETEC	47.2 ^a	36.5, 58.1									46	1			27	15																								
	Non-ETEC	32.7 ^b	27.3, 38.4									192	4			13	82																								
Trimethoprim	ETEC	69.7 ^a	59.0, 79.0								27						62																								
	Non-ETEC	31.6 ^b	26.3, 27.3								198	1					92																								

*The 95% confidence interval for the proportion of resistant isolates was calculated using the exact binomial method. **The MIC values give the concentration of amoxicillin. The concentration of clavulanic acid is half the concentration of amoxicillin. Different letters indicate a significant difference in the probability of isolates being resistant ($p < 0.05$), as tested by a generalised linear mixed model. Vertical solid lines indicate microbiological breakpoint values for antimicrobial resistance; vertical dashed lines indicate breakpoints for intermediate sensitivity. White fields represent the range of dilutions tested. MIC values below the lowest concentration tested are presented as one dilution step below the range. MIC values greater than the highest concentration in the range are presented as one dilution step above the range.

Table 3: Distribution of resistant isolates at herd level

Antimicrobial agent	Isolate Type	Herd 1		Herd 2		Herd 3	
		ETEC	NON-ETEC	ETEC	NON-ETEC	ETEC	NON-ETEC
Amoxicillin+Clavulanic acid		0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.6%)	0 (0.0%)	0 (0.0%)
Ampicillin		14 (33.3%)	7 (14.3%)	19 (86.4%)	2 (1.3%)	21 (84.0%)	0 (0.0%)
Apramycin		13 (31.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Ceftiofur		0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Ciprofloxacin		0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Colistin		0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Gentamicin		13 (31.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Neomycin		0 (0.0%)	1 (2.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (1.2%)
Sulphamethoxazole		41 (97.6%)	7 (14.3%)	0 (0.0%)	54 (34.4%)	21 (84.0%)	68 (80.0%)
Spectinomycin		16 (38.1%)	2 (4.1%)	0 (0.0%)	42 (26.8%)	0 (0.0%)	18 (21.2%)
Streptomycin		18 (42.9%)	9 (18.4%)	0 (0.0%)	62 (39.5%)	13 (52.0%)	59 (69.4%)
Tetracycline		42 (100.0%)	18 (36.7%)	0 (0.0%)	50 (31.9%)	0 (0.0%)	27 (31.8%)
Trimethoprim		41 (97.6%)	6 (12.2%)	0 (0.0%)	34 (21.7%)	21 (84.0%)	52 (61.2%)
Total		42	49	22	157	25	85

Table 4: Profile of antimicrobial resistance in *E. coli* isolates in faecal samples from weaned pigs and pen floors

Type	Pattern	ETEC isolates	Non-ETEC isolates
28	AMP, APR, GEN, SMX, SPE, STR, TET, TMP	13 (14.6%)	
27	AMP, NEO, SMX, SPE, STR, TET, TMP		1 (0.3%)
26	AMP, SMX, SPE, STR, TET, TMP		7 (2.4%)
25	SMX, SPE, STR, TET, TMP	1 (8.1%)	
24	AMP, SMX, STR, TET, TMP		38 (13.1%)
23	AMP, SMX, SPE, STR, TMP		6 (2.1%)
22	AMP, SMX, SPE, STR, TET		1 (0.3%)
21	SMX, STR, TET, TMP	4 (4.5%)	
20	SMX, SPE, TET, TMP	2 (2.2%)	
19	SMX, SPE, STR, TET		14 (4.8%)
18	AMP, SMX, TET, TMP		5 (1.7%)
17	AMP, SMX, STR, TMP	13 (14.6%)	16 (5.5%)
16	AMP, SMX, SPE, STR		8 (2.7%)
15	SPE, STR, TMP		4 (1.4%)
14	SPE, STR, TET		9 (3.1%)
13	SMX, TET, TMP	21 (23.6%)	8 (2.7%)
12	AMP, STR, TET		1 (0.3%)
11	AMP, SPE, STR		4 (1.4%)
10	AMP, SMX, TMP	8 (9.0%)	3 (1.0%)
9	AMP, SMX, STR		19 (6.5%)
8	STR, TET		1 (0.3%)
7	GEN, SMX		2 (0.7%)
6	APR, STR		1 (0.3%)
5	AMP, TET	1 (8.1%)	
4	AMP, AUC		1 (0.3%)
3	TET		21 (7.2%)
2	SMX		1 (0.3%)
1	AMP	19 (21.3%)	10 (3.4%)
0	Fully susceptible	7 ^a (7.9%)	110 ^b (37.8%)
Total		89	291

Notes: For abbreviations, refer to Table 1. Isolates where no resistance was observed were labelled “fully susceptible”.

Different letters indicate a significant difference in the probability of isolates being fully susceptible ($p < 0.05$) as tested by a generalised linear mixed model.

Table 5: Resistance patterns in ETEC isolates detected from pig and pen floor samples in the same pen

Herd Id	Pen Id	Pig isolates						Pen floor isolates				
1	1	21	20	13	13	13	13	21	20	13	13	13
1	2	13	13					25	21	13	13	
1	3	13	13	13	13	13		21	13	13	13	13
1	4	28	28	28	28			28	28	28	28	13
1	5	28	28	5				28	28	28		
2	6	1	1					1	1	1	1	1
2	7	1	1	1	1			1	1	1		
3	8	17	17	17	10	10	0	17	17	17	10	
3	9	17	10	10	0			10	10	10		
3	10	17						17	17	17	17	17

For each pen, one to two ETEC were isolated from one to three diarrhoeic pigs. For each pen floor sample, one to five ETEC were isolated. The colour represents the resistance pattern given by the corresponding Type number in Table 4.

Table 6: Agreement in the resistance classifications of ETEC isolates for selected antimicrobial agents between pig and pen isolates

Antimicrobial agent ^a	Within-sample agreement		Resistance classification agreement	
	Pig sample ^b	Pen sample ^c	Agreement between pigs within the same pen ^d	Agreement between pig and corresponding pen floor isolates ^e
Ampicillin	17/18	10/11	6/7	10/10
Apramycin	17/18	10/11	7/7	10/10
Gentamycin	17/18	10/11	7/7	10/10
Spectinomycin	16/18	8/11	7/7	9/10
Streptomycin	15/18	6/11	6/7	7/10
Sulphamethoxazole	16/18	11/11	6/7	10/10
Tetracycline	18/18	11/11	7/7	10/10
Trimethoprim	16/18	11/11	6/7	10/10

^a Selected antimicrobial agents with an overall resistance rate >1%. ^b Comparison of isolates from 18 diarrhoeic pigs where multiple ETEC were isolated. ^c Comparison of isolates from 11 pen floor samples where multiple ETEC were isolated. ^d A pig was classified as resistance positive for a specific antimicrobial class if one or more ETEC isolates were resistant. ^e **Pig resistance:** A pen was classified as resistance positive for a specific antimicrobial class if a minimum of one ETEC isolate from one or more pigs in the pen was resistant. **Pen floor resistance:** Pen floor samples were classified as resistance positive if a minimum of one ETEC isolate from the sample was resistant.

7 Discussion

The purpose of this PhD project was to develop new objective methods for assessing porcine intestinal health at batch level. The focus in this project has been on improving diagnostic methods and antimicrobial treatment regimens of intestinal diseases in nursery pigs, which can lead to more precise and potentially reduced antimicrobial usage. Replacing and rethinking the need for antimicrobial usage in order to achieve prudent use has not been part of this project, but should be taken into consideration when forming conclusion on the results.

7.1 Discussion of materials & methods

All data included in this thesis were based on observations and samples from Danish nursery pig facilities.

Materials & methods of Study 1

The aim of this study was to determine the within-herd prevalence of diarrhoea and intestinal pathogens in batches of nursery pigs that had no signs of intestinal disease and therefore did not require treatment. The included herds had previously been used in studies of diarrhoea in nursery pigs (Pedersen et al., 2014a). The herds were reused due to the willingness of herd owners to participate in an additional study. Furthermore, by investigating the same herds, the diarrhoea prevalence assessed in apparently clinically healthy batches could be compared to that of batches with clinical outbreaks of diarrhoea. A cross-sectional study design was chosen and the study herds were believed to be representative of most Danish herds with intensively managed nursery units. In the 16 included study herds, a large variation was observed in health status, herd size, productivity and antimicrobial use. No information on management and dietary factors was included in the analysis, but it is possible that several of these factors could have had an influence on the conclusion of the study. The prevalence of intestinal pathogens was determined by qPCR testing of faecal samples from a sample size of 256 pigs. The relatively small sample size was chosen mainly due to economic reasons. The tested faecal samples were randomly selected and samples from all 16 herds were represented in the analysis. However, due to the small sample size, the within-herd prevalence of intestinal pathogens might vary from the prevalence determined in this study.

Materials & methods of Study 2

The aim of this study was to improve the effect of antimicrobial treatment for intestinal infections in groups of nursery pigs. A field trial was selected to reflect “real life” scenarios, and two different antimicrobials and four treatment strategies were tested under farm conditions. The type of antimicrobial and the treatment strategies tested in the trial were relevant to common pig practice, and results derived from the trial can therefore be used to advise pig practitioners on the treatment of intestinal infections. A disadvantage of using field trials is that many factors that can influence the results are uncontrolled. In this study, three herds were selected by common SPF status, use of standard feeding manuals and presence of high-pathogenic diarrhoea outbreaks in nursery pigs. However, management factors were not controlled and these could be different among the herds. Pigs challenged with known doses of different pathogens could be tested under experimental conditions in laboratory facilities, and the effect of different types of antimicrobial and the time of treatment on daily weight gain could be determined with higher certainty. We observed a difference in diarrhoea prevalence and pathogen detection at different time points at batch and herd level. These within- and between-herd variations in clinical diarrhoea and infection dynamics have previously been reported from investigations of intestinal diseases in nursery pigs (Moller et al., 1998; Stege et al., 2004). Therefore, if treatment studies are to be performed under controlled conditions, these observed variations at different time-points should be incorporated, resulting in a large and costly study set-up.

Pen was the unit of randomisation, and eight pens were included in each batch. The disadvantage of using pen level for randomisation was the risk of herd personnel spreading manure – and thereby potential intestinal pathogens – between study pens. The advantage was that the sample size of pigs was reduced, and due to economic constraints, pen-level randomisation was chosen.

The study had a 2x4 factorial design and pens were allocated to four different treatment strategy groups, none of which were defined as controls in Paper II. Pens selected for treatment strategy 4 were only treated if a clinical outbreak of diarrhoea occurred, which happened in three of 16 pens. This type of strategy – where clinical inspection criteria are used to determine the time-point at which antimicrobial treatment for PIDC should be initiated in a batch of pigs – is the most common strategy used in Denmark, and it was therefore considered to be a control group in this study (Pedersen et al., 2015). Due to animal welfare considerations, all pens were treated when a diarrhoea outbreak occurred, regardless of the predetermined time-point for treatment. This resulted in different time-points of treatment within each strategy group. Therefore, the conclusions derived

from this study concern the effect of different treatment strategies and not the different time-points of treatment initiation on daily weight gain.

Antimicrobial treatment at pen level was administered via water troughs. Due to legal regulations, all pigs had free access to fresh water via drinking nipples during the treatment period. Therefore, there was a risk that not all pigs in the pen drank the medicated water, and a uniform dose of antimicrobials could not be guaranteed for all pigs. Administration via the drinking water system might have been preferable, but this was not possible in the selected study herds.

To analyse the effect of antimicrobial type and treatment strategy on daily weight gain, mixed linear models were used with herd, batch and pen as random effects. In Herd A, the ADG was significantly lower than in Herds B and C. Statistical linear models from each of the three herds were built to investigate herd differences. The estimates from the reduced models showed patterns similar to the full model, and the herd variable was therefore included as a random rather than fixed effect in the final model.

According to the sample size calculations, 12 batches should have been included in the study. Due to a fire in Herd C during the trial, two batches were excluded, giving a total of 10 included batches. It is likely that the reduced sample size had an impact on the statistical power of the study, but no additional power calculations were performed on the reduced sample size.

Materials & methods of Study 3

The aim of this study was to investigate whether testing of faecal pen floor samples can be used both to identify ETEC-positive diarrhoeic pigs and for susceptibility testing.

Pen was included in the study when the pen-level prevalence of clinical diarrhoea assessed in randomly selected indicator pigs was above 25%. Pens were therefore not randomly selected, but included at a time-point when the inclusion criteria were fulfilled. Pens from one batch in Herd A, four batches in Herd B and two batches in Herd C were included. This resulted in an unbalanced distribution of isolates among the three herds. No further analysis of the effect of herd on ETEC detection and susceptibility testing was performed.

The sampling procedure in Study 3 was based on the isolation of *E. coli* colonies with or without haemolytic activity after culture of faecal samples from three diarrhoeic pigs per pen, in order to establish the ETEC status of all pigs within the same pen. When present after culture, a maximum of two isolates with haemolytic activity and two non-haemolytic isolates from each pig were

examined. Furthermore, a maximum of five haemolytic, and five non-haemolytic isolates per pen floor sample were examined to establish the ETEC status of the pen floor sample. The selected sample size was chosen partly for convenience and economic factors related to the laboratory analysis, and to simulate the commonly used diagnostic approach of Danish veterinary practitioners.

The method used to compare ETEC status and resistance of isolates from pig and pen floor samples was based on commercially available techniques (phenotypes, haemolytic activity and virulence gene detection) and standard panels of relevant antimicrobial agents used by diagnostic veterinary laboratories. Other DNA-based techniques (such as pulsed-field gel electrophoresis) could have been used to make the comparison of isolates from pigs and pen floor samples more precise and could have made it possible to evaluate whether the isolates from pig and pen floor samples were identical. The aim of this study was to investigate whether the diagnosis and the antimicrobial selection would be the same when both pen floor isolates and samples from diarrhoeic pigs were used. Standard commercial methods were therefore used in this study instead of advanced DNA techniques. The clinical breakpoints used to interpret MIC values were a combination of CLSI-approved breakpoints and breakpoints routinely used by the Laboratory of Swine diseases, Kjøllerup, Denmark and the Danish Veterinary Institute. The lack of approved clinical breakpoints for several of the tested antimicrobials could have resulted in misclassification of the susceptibility status of the isolates used in Study 3. The same clinical breakpoint was used when the resistance status of isolates from pig and pen floor samples were compared, and although the true resistance status might be questionable, a comparison was considered to be reasonable.

7.2 Discussion of hypothesis 1: New objective methods to identify intestinal infections in nursery pigs are necessary to ensure the prudent use of antimicrobials

In Study 1, the diarrhoea prevalence observed in batches of non-medicated nursery pigs considered by the herd personnel to be healthy was surprisingly high, at 32.6% on average (Paper I). A previous study of batches from the same study herds showed a diarrhoea prevalence of 33% in batches where the herd personnel initiated antimicrobial batch medication (Pedersen et al., 2014a). This shows that in the study herds included in Study 1, the diarrhoea prevalence was equal in batches assessed to be healthy and in batches assessed to be diseased and in need of antimicrobial treatment. The clinical examinations revealed that very few pigs had clinical signs indicating diarrhoea (hyperaemic anal region/faecal staining of hindparts). These results show

that assessing diarrhoea prevalence by visual inspection of batches of nursery pigs will lead to a considerable underestimation of the actual occurrence clinical diarrhoea. Furthermore, inspection of the pen floors revealed that there were few diarrhoeic faecal droppings that could indicate the presence of diarrhoeic pigs in the pen. These results are relevant because the current procedure involves decisions being made on batch medication for intestinal infection in nursery pigs based on indications of diarrhoea and counting diarrhoeic faecal droppings on the pen floor. This procedure will lead to a considerable lack of treatment for pigs with subclinical diarrhoea.

Study 1 also revealed no relationship between the diarrhoea status of the individual pig and the presence of intestinal pathogens detected by qPCR in faecal samples. Furthermore, no intestinal pathogens were detected in a high proportion of pigs with clinical diarrhoea. The most frequently detected pathogen was *L. intracellularis*, and high levels of *L. intracellularis* indicative of PE were detected in 44% of the tested pigs (Pedersen et al., 2012c; Johansen et al., 2013; Pedersen et al., 2013b; Collins and Barchia, 2014). The excretion levels of the other analysed pathogens were at the same level as those previously reported from diarrhoeic nursery pigs (Stahl et al., 2011).

These results show that the diarrhoea status of the individual pig is a poor indicator of intestinal infection, and that subclinical infections are common. Using the diarrhoea status of the individual pig as the sole diagnostic method will lead to the treatment of pigs with no intestinal pathogens, as well as a lack of treatment for pigs with subclinical intestinal infections.

The results of Study 1 revealed that decisions on batch medication made by assessing the number of pigs with faecal staining, counting diarrhoeic faecal droppings on the floor or determining the diarrhoea status of the individual pig are likely to result in a sizeable proportion of pigs with diarrhoea not receiving treatment, and diarrhoeic pigs with no intestinal infections receiving unnecessary treatment. Therefore, clinical diagnosis alone is inadequate in ensuring the prudent use of antimicrobials, i.e. where treatments are precise and only used in cases of diarrhoea caused by bacterial pathogens.

7.3 Discussion of hypothesis 2: Efficacy of batch treatment depends on the correct timing and choice of antimicrobial

Timing of treatment

The primary effect of antimicrobial treatment for intestinal infections was assessed using the ADG in this thesis. A total of four different treatment strategies were tested in Study 2. The selection of treatment strategies was based on the relevance of treatment regimens for PIDC in Danish pig production. The main finding of Study 2 was that ADG was associated with treatment strategy, and the earlier the treatments were initiated post weaning, the higher the ADG. In contrast, the diarrhoea prevalence at pen level and the excretion level of intestinal bacterial pathogens were observed to increase over time. These results correspond well with the results from Study 1, where diarrhoea prevalence observed at pen level increased with the age of the pigs, which could be due to a higher stocking density resulting in increased infection pressure, a longer time at risk for developing diarrhoea and different diets (Funk et al., 2001).

In Study 2, 13 pens did not receive any antimicrobial pen treatment during the trial. These pens had the lowest ADG and the highest diarrhoea prevalence at the end of the study. These pens can be considered as a control group, and show the importance of adequate treatment for intestinal infections in nursery pigs.

Selection of antimicrobial agent

The two most common antimicrobial agents used for treating intestinal diseases in Denmark (tylosin and doxycycline) were used in Study 2 at the recommended dose and a treatment duration of 5 days. The effect of antimicrobial agent type on the ADG, diarrhoea prevalence and pathogenic bacterial load was assessed. The type of antimicrobial used for treatment did not have a statistically significant impact on ADG, whereas the diarrhoea prevalence at the end of the study was significantly higher in pens treated with tylosin compared to pens treated with doxycycline. The excretion level of pathogenic bacteria 2 days after the final day of treatment was used to evaluate the effect of type of antimicrobial on pathogenic bacteria. Doxycycline was found to be more efficient in reducing the excretion level of *L. intracellularis* compared to tylosin. Significantly higher levels of *L. intracellularis* were observed in floor samples 2 days after treatment in pens treated with tylosin compared to doxycycline.

This result was somewhat surprising due to a previous study showing treatment with tylosin reducing clinical signs and pathological lesions, and improving the growth rate in nursery pigs

challenged with *L. intracellularis* (Paradis et al., 2004). Furthermore, tylosin has shown high intracellular and extracellular activity against *L. intracellularis* (Wattanaphansak et al., 2009; Yeh et al., 2011). In this trial, a recommended dose of 7.5 mg per kg bodyweight was used for a period of 5 days. The results indicate that higher doses of tylosin or a longer treatment period may be necessary when treating pigs with *L. intracellularis* infections.

The effect on the excretion level of ETEC could not be evaluated because an equal reduction in ETEC excretion was observed in pens treated with either doxycycline or tylosin and pens not receiving any antimicrobial treatment. This indicates that it is possible for pigs to stop excreting ETEC without any antimicrobial treatment. A similar finding of reduced excretion levels in non-vaccinated and untreated control pigs was reported in a vaccine trial, in which nursery pigs were challenged with ETEC (Tiels et al., 2008). Therefore, measures other than the excretion level 2 days after the last treatment could be used to evaluate the effect of antimicrobial ETEC treatment. The effect of antimicrobial agent on the excretion of *B. pilosicoli* could not be evaluated because *B. pilosicoli* was a rare finding in the three study herds.

Fixed doses and a treatment length of 5 days were used in Study 2. Conclusions on the effect of doxycycline or tylosin treatment on ADG, diarrhoea prevalence and excretion of pathogenic bacteria might have been different if higher doses or a longer treatment length were used. The doses and treatment length used in this study were chosen because they are the most commonly used by Danish pig practitioners for the treatment of diarrhoea outbreaks in nursery pigs with unknown infectious pathogen (Pedersen et al., 2015).

7.4 Discussion of hypothesis 3: Testing of pen floor samples may enhance the prudent use of antimicrobials for batch treatment of intestinal infections in nursery pigs

The diagnostic value of testing pen floor samples on the day of antimicrobial treatment initiation

In Study 2, the effect on ADG of detecting intestinal pathogens at treatment initiation was demonstrated in pigs treated on Day 14 post weaning. The highest ADG was achieved when the pen floor sample was positive for one or more of the four examined pathogens on the day of treatment initiation. In other words, pigs with a negative pen floor sample did not benefit from the antimicrobial treatment as much as pigs with a positive pen floor sample. The ADG of pigs treated on Day 14 that had no detectable pathogens was similar to that of pigs treated on Day 28 or later,

which may support the idea that these pens were treated before an infection occurred in the pigs. Overall, these findings show that antimicrobial treatment had the greatest effect on ADG in the pens where pigs excreting intestinal pathogens were treated early, when clinical diarrhoea was still at a low level. The presence of subclinical infections detected in Study 2 corresponds with the results from Study 1, showing that subclinical intestinal infections were common, in agreement with previous reports (Jacobson et al., 2003; Collins and Barchia, 2014).

These results could have an impact on achieving prudent antimicrobial usage when treating outbreaks of PIDC. If treatments are initiated solely by choice of strategy, the recommendation would be to treat the pigs early, before clinical signs of diarrhoea become evident. A consequence of this recommendation is that all pigs would be treated soon after weaning, and the use of antimicrobials would increase. Therefore, the decision to initiate treatment should be based on the real-time examination of pen floor samples in pens where no clinical signs are evident. The result of the pen floor examination should indicate whether or not intestinal pathogens are present, and whether or not antimicrobial treatment is necessary. Due to the small sample size in Study 2, it was not possible to investigate the effect of different excretion levels of pathogenic bacteria on ADG, which would have determined a critical threshold of pathogenic bacteria excretion at which antimicrobial treatment could be recommended. If this approach for initiating batch medication for PIDC is to become widely used, a cheap, fast and easy qPCR method must be developed and tested in field trials, where the decisions about treatment are based on real-time pen floor analysis of intestinal pathogen excretion levels at herd facilities.

Drug selection based on susceptibility testing of pen floor isolates

This thesis has examined the presence of ETEC in pen floor samples and in faecal samples from diarrhoeic pigs (Study 3). The primary reason that ETEC was chosen for this study among the intestinal pathogens included in the thesis is that *E. coli* are easy to culture by routine methods and virulence gene detection by PCR analysis is regularly used in veterinary diagnostic laboratories. In addition, ETEC was frequently detected in the three study herds from Study 2, making it possible to conduct a reasonable number of comparisons. The results showed that the same pathotype of ETEC was simultaneously present in diarrhoeic pigs and on the pen floor where the pigs were housed (Paper III). Furthermore, the resistance pattern was also comparable in isolates from pigs and pen floor samples (Paper IV). Although the sample size of the examined pens in Study 3 was quite low and only included isolates from three herds, the conclusions of the study suggest a new approach to diagnosing ETEC-infected pigs, by

examining isolates from pen floor samples instead of samples from individual diarrhoeic pigs, which would be highly relevant in cases of colibacillosis outbreak.

In relation to PIDC, a suggested approach could be to culture and carry out susceptibility testing of isolates from pen floor samples found positive for ETEC genes by multiplex qPCR. This approach would ease the diagnostic work of pig practitioners during diarrhoea outbreaks in nursery pigs, where the causative agent can be determined by multiplex qPCR analysis, and a susceptibility test can guide the selection of the antimicrobial agent used for treatment. If culture and/or susceptibility testing of *L. intracellularis* and *B. pilosicoli* become routinely available in future, the study should be repeated to investigate whether the same relationship exists between the presence of *L. intracellularis* or *B. pilosicoli* and similar resistance patterns in diarrhoeic pigs and on the pen floor as was found with ETEC.

7.5 Discussion of hypothesis 4: Intestinal infections in batches of nursery pigs are often of mixed aetiology

In both Study 1 and 2, pathogen detection was performed by qPCR analysis of faecal samples from individual pigs (Paper I) and pen floor samples (Paper II). The multiplex qPCR analysis used in this thesis was commercially available and performed by the Danish Veterinary Laboratory. As previously described, bacterial infections by ETEC, *L. intracellularis* and *B. pilosicoli* are considered to be the main intestinal pathogens in Danish nursery pigs, and viral infections have not been shown to have any influence. For this reason, no further analysis of other intestinal pathogens of bacterial, viral or parasitic origin was performed in this study. An additional *E. coli* examination of individual diarrhoeic pigs by bacterial culture and virulence gene detection was performed in Study 3 (Paper III).

In both Study 1 and 2, ETEC and *L. intracellularis* were the most frequently detected pathogens. In Study 1, the detected level of adhesin genes F4 and F18 was equal, while F18 was the dominant adhesin gene detected in Study 2. The reason that F4 genes were more frequently detected in Study 1 compared to Study 2 could be that Study 1 included samples from 16 herds, whereas Study 2 only included three herds. There was a clear pattern of pathogen detection related to age in both studies. ETEC were most frequently detected in samples from young pigs or samples from the floor of pens containing young pigs, whereas *L. intracellularis* and *B. pilosicoli* were more frequently detected in samples from older pigs or samples from the floor of pens containing older pigs. This finding

corresponds well with previously published results on the typical age of pigs infected with ETEC, *L. intracellularis* and *B. pilosicoli* (Wills, 2000; Stege et al., 2004; Fairbrother et al., 2005).

As previously defined, PIDC describes diseases of multiple infectious aetiology affecting the small and large intestines, causing clinical diseases and failure to gain weight. Pathogen detection in Study 1 was based on randomly selected pigs with and without diarrhoea. Mixed infections (PIDC) were detected in 26% (32/121) of pig samples where pathogens were detected. At pen level, PIDC was detected in 35% (27/77) of pens where pathogens were detected in Study 1. In Study 2, PIDC was detected at pen level in 22% (29/131) of pens. At batch level, PIDC was detected in 50% (21/42) of pathogen-positive batches in Study 1, and 51% (19/37) of pathogen-positive batches in Study 2. These results show that PIDC was frequently detected at pig, pen and batch level, which must be considered when diagnosing intestinal diseases in nursery pigs and when decisions involving antimicrobial treatment, vaccination programmes or dietary changes are being made.

The results from Study 1 and Study 2 show the importance of using microbiological testing in combination with clinical signs when diagnosing PIDC. The diarrhoea status of the individual pig was a poor indicator of intestinal infections, and multiple intestinal pathogens were present in batches of healthy pens. Therefore, when diagnosing PIDC, clinical diagnosis alone is insufficient, and multiplex qPCR analyses of faeces from pen floor samples can be used to diagnose the causative infectious agent(s) involved in the diarrhoea outbreak. However, due to the large within-herd variation in diarrhoea prevalence and pathogen detection observed in Study 1 and 2, several diarrhoea outbreaks should be examined by qPCR analysis to ensure a precise diagnosis at batch level. This finding confirms the previously published results on the usefulness of pen floor sampling for diagnosing intestinal diseases in nursery pigs (Pedersen et al., 2015).

8 Conclusion

Conclusion on hypothesis 1: New objective methods to identify intestinal infections in nursery pigs are necessary to ensure the prudent use of antimicrobials

Based on the results from Study 1, the first hypothesis can be confirmed, since new objective methods to identify intestinal infections in nursery pigs are necessary to ensure the prudent use of antimicrobials. The results of this thesis show that diarrhoea is highly prevalent in nursery pigs and can be present without any other clinical signs. No relationship was identified between the diarrhoea status of the individual pig and the presence of intestinal pathogens detected by qPCR in faecal samples.

Conclusion on hypothesis 2: Efficacy of batch treatment depends on the correct timing and choice of antimicrobial

Based on the results from Study 2, the second hypothesis can be confirmed. The efficacy of batch treatment is highly dependent on correct timing. The earlier the pigs were treated, the higher the average daily weight gain.

The most efficient treatment strategy resulting in the highest average daily weight gain was treatment at pen level 14 days after weaning. Treatment with doxycycline was more effective than treatment with tylosine in reducing *L. intracellularis* excretion and clinical diarrhoea after treatment.

Conclusion on hypothesis 3: Testing of pen floor samples may enhance the prudent use of antimicrobials for batch treatment of intestinal infections in nursery pigs

Based on the results from Study 2 and Study 3, the third hypothesis can be confirmed. Pen floor samples can be tested for intestinal pathogens to identify groups of subclinically infected pigs, and to indicate where antimicrobial treatment can be recommended and where it can be avoided. Furthermore, an association was identified between isolates from diarrhoeic nursery pigs and samples from pen floor samples in terms of both ETEC detection and resistance patterns. Based on this, a new sampling method for analysing isolates from pen floor samples is suggested for diagnosing ETEC-positive pigs and determining resistance profiles for the selection of antimicrobial agents.

Conclusion on hypothesis 4: Intestinal infections in batches of nursery pigs are often of mixed aetiology

Based on the results from Studies 1, 2 and 3, the fourth hypothesis can be confirmed. In this thesis, the presence of multiple intestinal pathogens was observed at pig, pen, batch and herd level. The term Porcine Intestinal Disease Complex (PIDC) is suggested to describe diseases of multiple aetiology affecting the small and large intestines, causing clinical diseases and failure to gain weight.

Overall conclusion

Intestinal infections in groups of pigs before clinical signs are evident may be diagnosed using pooled pen floor samples tested by multiplex qPCR, which can be used to support the decision for antimicrobial treatment initiation. The advantages of diagnosing intestinal infections using this method include better productivity and the avoidance of unnecessary antimicrobial treatments. A possible reduction in antimicrobial usage can therefore be achieved, with the benefit of reduced medicine costs and a reduced risk of antimicrobial resistance developing.

9 Implications and perspectives

9.1 Future perspectives

The results from this thesis have contributed new knowledge about the diagnosis and treatment of PIDC in nursery pigs. The results emphasise the need to use new diagnostic methods for assessing the intestinal health of groups of nursery pigs when deciding on antimicrobial batch treatment. To achieve a prudent use of antimicrobials, clinical diagnosis of intestinal disease in nursery pigs must be supplemented with laboratory analysis. This thesis has demonstrated the diagnostic value of qPCR testing of faecal pen floor samples at the time of treatment, but a critical threshold of pathogenic bacteria excretion has not been determined. Field trials, where antimicrobial treatment is initiated at different excretion levels of pathogenic bacteria, must therefore be conducted to investigate whether a critical threshold can be determined.

The presence of PIDC must be considered when diagnosing and treating diarrhoeic nursery pigs, and the polymicrobial nature of PIDC should be taken into account when choosing diagnostic methods. Multiplex qPCR testing of relevant intestinal pathogens is therefore recommended. Furthermore, the presence of PIDC should be considered when future guidelines for the selection of antimicrobial agents for intestinal disease in pigs are developed.

9.2 Practical importance of the results

The results of Study 3 showed that ETEC isolates from pen floor samples can be used for diagnosing ETEC-positive diarrhoeic nursery pigs and for susceptibility testing of ETEC isolates for antimicrobial selection. As a consequence of these results, the Danish Veterinary Institute has implemented susceptibility testing of ETEC isolates from pen floor samples, and this is now a commercially available method for pig practitioners in Denmark.

The growing demands of reducing antimicrobial usage in pig production will result in an increase in the need for precise evidence-based diagnostic methods. If commercial methods for pen-level qPCR testing become available at affordable rates, testing of pen floor samples could become a common method to support the decision for initiating antimicrobial treatment of intestinal diseases.

10 References

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